The action of drugs on potassium-evoked release of acetylcholine from rat hippocampal prisms with special reference to tacrine and classical cholinesterase inhibitors.

Amerasinghe, Nirosha Devika

THE ACTION OF DRUGS ON POTASSIUM-EVOKED RELEASE OF ACETYLCHOLINE FROM RAT HIPPOCAMPAL PRISMS WITH SPECIAL REFERENCE TO TACRINE AND CLASSICAL CHOLINESTERASE INHIBITORS

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NIROSHA DEVIKA AMERASINGHE

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Department of Pharmacology

King's College

Manresa Road

Chelsea

London SW3 6LX

ABSTRACT

Therapeutic strategies for the symptomatic treatment of Alzheimer patients have mostly been targeted at improving the central cholinergic deficit, which is correlated to the degree of cognitive impairment. One such strategy which increases the availability of acetylcholine (ACh) has been the use of cholinesterase (ChE)-inhibitors, among which tacrine, has been claimed to cause substantial improvement in cognitive function. The present study investigated the effects of tacrine and the more classical ChE-inhibitors eserine, neostigmine and edrophonium *in vitro*. The K^+-evoked release of [^{3}H]ACh was measured from rat hippocampal prisms preloaded with [^{3}H]choline.

Contrary to several previous reports, each of these classical ChE-inhibitors potentiated K^+-evoked release at 10-200μM concentrations. Tacrine caused comparable potentiation although concentrations exceeding 100μM gave marked inhibition of [^{3}H]ACh release. Potentiation of release was observed even in the presence of the choline uptake-inhibitor hemicholinium-3 (HC-3), suggesting that the observed effects were due in part, to a mechanism additional to that of inhibition of cholinesterases.

Since tacrine and the other ChE-inhibitors have been reported to have PCP-like activity, the benzomorphan σ ligands (+)cyclazocine or (±)pentazocine were tested and found to oppose potentiation without affecting control K^+-evoked release. However, negligible displacement of [^{3}H](+)pentazocine binding from hippocampal membranes by the ChE-inhibitors, indicated that the potentiation is unlikely to involve benzomorphan-like σ binding. The non benzomorphan σ ligands haloperidol and DTG inhibited control K^+-evoked release as markedly as did tacrine, suggesting a role for σ binding in the inhibition of [^{3}H]ACh release.

The possibilty that potentiation of [^{3}H]ACh release might involve muscarinic or nicotinic receptor-mediated feedback was investigated since the ChE-inhibitors, and cyclazocine, pentazocine and (+)NANM are also known to displace nicotinic and muscarinic receptor binding. The muscarinic antagonist atropine enhanced the potentiation caused by the ChE-inhibitors, supporting the existence of a muscarinic receptor mediating negative feedback. However, the nicotinic antagonist dihydro-β-erythroidine (DHβE) had no effect, although acting alone, it potentiated K^+-evoked [^{3}H]ACh release. This latter effect was similar to that of the ChE-inhibitors and was similarly blocked by (+)cyclazocine and (±)pentazocine. It is suggested that the ChE-inhibitors and DHβE block negative feedback mediated by nicotinic autoreceptors and unmask the σ inhibitory action of (+)cyclazocine and (±)pentazocine. However (+)NANM which was found to be a muscarinic autoreceptor antagonist, additionally required atropine to unmask its σ inhibitory effect. The micromolar concentrations of σ ligands required to inhibit [^{3}H]ACh release suggest the involvement of low affinity σ binding sites.

SUMMARY

Chapter 3

- HC-3 increases the S2/S1 ratio of K^+-evoked [^{3}H]ACh release from rat hippocampal prisms by a mechanism best explained by block of choline uptake rather than by antagonism at muscarinic receptors.
- Tacrine and the classical ChE-inhibitors eserine, neostigmine and edrophonium caused a further increase in the S2/S1 ratio over and above the increase caused by HC-3. This implies that a mechanism exists for the potentiation of the release of [^{3}H]ACh in addition to ChE inhibition.
- Tacrine differs from the classical ChE-inhibitors however, in causing inhibition of [^{3}H]ACh release at concentrations greater than 50μM. This cannot be the result of negative feedback mediated by the action of ACh at muscarinic autoreceptors. Neither can it be due to the influence of monoamines or 5-HT (which are also released by tacrine), since prior reserpinization of the slices did not change the dual effects of tacrine.
- Tacrine (as well as the other ChE-inhibitors) in the concentration range affecting [^{3}H]ACh release, caused appreciable displacement of [^{3}H]QNB from hippocampal membrane preparations. Direct actions therefore, at muscarinic autoreceptors cannot be excluded at this stage.
- The above findings clarify the contradictory literature on the effects of tacrine and the classical ChE-inhibitors.

Chapter 4

- Since tacrine is also known to interact with PCP binding sites, the effects of the non-competitive NMDA antagonists PCP and MK-801 were tested. MK-801 had no effect on K^+-evoked release or on the neostigmine-induced potentiation of [^{3}H]ACh release. PCP however, (which is also known to bind σ binding sites) caused an increase in [^{3}H]ACh release at 100μM concentrations.

- The effects were investigated of σ ligands on K^+-evoked release and on the ChE-inhibitor-induced potentiation of [^{3}H]ACh release. The benzomorphan σ1 selective ligands (+)cyclazocine and (±)pentazocine opposed the ChE-inhibitor-induced potentiation of [^{3}H]ACh release at concentrations which alone had no effect on control K^+-evoked release.
- However the potentiation of release caused by neostigmine was not inhibited by the "prototypic" σ ligand (+)NANM at concentrations comparable to that of (+)cyclazocine.
- The ChE-inhibitors eserine, neostigmine and edrophonium caused only weak displacement of the σ1 radioligand [^{3}H](+)pentazocine from hippocampal membrane preparations. This suggests that the potentiation of [^{3}H]ACh release by the ChE-inhibitors is unlikely to be due to an interaction σ1 binding sites.
- The non benzomorphan σ ligands haloperidol, (+)3-PPP and DTG, which act at σ1 and σ2 sites were also investigated. Haloperidol and DTG were shown to inhibit control K^+-evoked [^{3}H]ACh release in a concentration-dependent manner and haloperidol was also observed to cause a functional antagonism of the potentiation caused by the ChE-inhibitors.
- It was provisionally considered therefore, that the inhibition of [^{3}H]ACh release caused by DTG, haloperidol, and also by tacrine, might be mediated by σ2 binding sites. However it was noted that micromolar concentrations were necessary in contrast to the nanomolar IC_{50} and K_D values previously reported from radioligand binding studies.
- The σ binding properties of tacrine and the effects described above of benzomorphan and non benzomorphan σ ligands on [^{3}H]ACh release and its potentiation, have not been reported previously.

Chapter 5

- The possibilty was examined that nicotinic or muscarinic actions of the benzomorphan σ ligands (+)cyclazocine, (±)pentazocine and (+)NANM and the

ChE-inhibitors are responsible for the different effects of these drugs on K^+-evoked [^{3}H]ACh release.

- In agreement with previous reports of muscarinic autoreceptors, atropine enhanced the potentiation of release caused by tacrine and the other ChE-inhibitors. It was argued that the ChE-inhibitors had minimal antagonist properties, if any, at the muscarinic autoreceptors and that [^{3}H]QNB displacement (Chapter 3) was not necessarily a reliable indicator of muscarinic autoreceptor binding.
- It was concluded that tacrine, acts as a muscarinic autoreceptor agonist, with maximum inhibitory effect at 30μM concentrations. However, an independent mechanism over-rides this with potentiation at this concentration (see below). At greater concentrations σ–mediated inhibition predominates (Chapter 4).
- The above interpretation of the effects of tacrine have not previously been suggested.
- The inhibition of K^+-evoked [^{3}H]ACh release by the muscarinic agonists oxotremorine or carbachol was antagonized by atropine and also by (+)NANM. These results support the role of muscarinic autoreceptors in the mediation of negative feedback and validate the superfusion release system used in this study.
- Although (+)NANM alone had no effect on the potentiation caused by neostigmine (Chapter 4), in the presence of atropine, the potentiation was opposed. It appears that atropine unmasks an inhibitory effect of (+)NANM, comparable to the overt effect of (+)cyclazocine and (±)pentazocine.
- The nicotinic agonists nicotine and DMPP had no effect on K^+-evoked [^{3}H]ACh release at 0.1-10μM concentrations.
- Although the competitive nicotinic antagonists DHβE and *d*-tubocurarine increased control K^+-evoked release of [^{3}H]ACh, DHβE had no effect over and above the ChE-inhibitor induced potentiation.
- It was suggested that DHβE and the ChE-inhibitors have a common mechanism of action. This was further supported by the observation that (+)cyclazocine or

(±)pentazocine but not (+)NANM, opposed the potentiation caused by DHβE, as well as the potentiation caused by the ChE-inhibitors (Chapter 4).

- It was proposed that nicotinic autoreceptors mediate negative feedback, by inactivating voltage operated Ca^{2+} channels at control K^+-evoked levels of ACh release. The ChE-inhibitors and DHβE block these autoreceptors, reactivate the Ca^{2+} channels and potentiate control [^{3}H]ACh release as observed.
- On this basis nicotine or DMPP might be expected to contribute to Ca^{2+} channel inactivation and to be "self-limiting" in their effects on K^+-evoked ACh release.
- The effect described above, of nicotinic autoreceptor blockade, on control K^+-evoked [^{3}H]ACh release have not been described previously.

Chapter 6

- The σ ligands (+)cyclazocine, (±)pentazocine and (+)NANM are also known to have antagonist actions at nicotinic receptors. Thus their lack of effect on control K^+-evoked release (Chapter 4), which contrasts with the marked effect of DHβE (Chapter 5), may be explained as the net result of potentiation mediated by the nicotinic autoreceptors and inhibition by the σ binding sites.
- In the presence of the ChE-inhibitors or DHβE the inhibitory effect of (+)cyclazocine or (±)pentazocine acting at the σ binding sites is unmasked. Since (+)NANM also blocks muscarinic autoreceptors however (Chapter 4), it additionally requires the presence of atropine to reveal its σ activity (Chapter 5).
- It is suggested that the inhibition caused by the benzomorphans (+)cyclazocine, (±)pentazocine and (+)NANM is similar to the functional antagonism observed with haloperidol and is mediated by an action at the σ binding sites.
- Inhibition of [^{3}H]ACh release was caused by micromolar concentrations of the σ ligands (Chapter 4), in contrast to their reported nanomolar IC_{50} and K_D values. It is speculated that the 'low affinity' σ binding sites are involved and noted that these have been implicated in the modulation of K^+ channels. Further work on this would be useful.

- It has not previously been recognized that σ binding opposes control and potentiated K^+-evoked [^{3}H]ACh release.

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"The first to present his case seems right,

till another comes forward and questions him."

Proverbs 18 vs 17

"Whatever exists has already been named,

and what man is has been known:

no man can contend with One Who is stronger than he."

Ecclesiastes 6 vs 10

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ABBREVIATIONS

~	Approximately
ACh	Acetylcholine
AChE	Acetylcholinesterase
APP	β Amyloid precursor protein
ChAT	Choline acetyltransferase
ChE-I	Cholinesterase inhibitor
Ca^{2+}	Calcium
Ci	Curie(s)
CNS	Central Nervous System
DA	Dopamine
DMPP	1,1-Dimethyl-4-phenylpiperazinium
DTG	1,3-Di(2-tolyl) guanidine
ECMA	Ethylcholine mustard aziridinium
g	Acceleration due to gravity
HC-3	Hemicholinium
5-HT	5-hydroxytryptamine
i.v	intravenous
IC_{50}	Concentration causing 50% inhibition
K^+	Potassium
K_i	Affinity constant for inhibitor
LDH	Lactate dehydrogenase
min	Minute(s)
MK-801	(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate
NA	Noradrenaline
(+)NANM	N-Allylnormetazocine
NMDA	N-methyl-*D*-aspartate

NMJ	Neuromuscular junction
PCP	Phencyclidine
(+)3-PPP	(+)-3-(3Hydroxyphenyl)-N-propyl piperidine
QNB	Quinuclidinyl benzilate
rpm	Revolutions per minute
S2/S1	Ratio of the 1st K^+ stimulation to the 2nd stimulation
SEM	Standard error of mean
w/v	Weight to volume
°C	Degrees Celsius

CHAPTER 1

General Introduction

Alzheimer's disease 'a neurodegenerative disorder' is one of the most devastating diseases of the human brain that affects many lives all over the world. It is the commonest cause of dementia amongst the elderly and the fourth major cause of death in the developed world, after heart disease, cancer and stroke (Van Dujin *et al.*,1991). Although first described in 1907 by the psychiatrist Alois Alzheimer as an exclusively presenile disorder, the senile form of the condition, which has been shown to be pathologically similar in the majority of the cases to the young demented patients, is now generally accepted as senile dementia of the Alzheimer type (SDAT). There are a number of different types of dementia of which dementia of the Alzheimer's type is the most common, accounting for over 50% of the cases (Bondareff, 1994).

Clinical symptoms and diagnosis of Alzheimer's disease

Since there is no effective treatment at present to prevent or ameliorate the progression of Alzheimer's disease, brain biopsy is not considered justified as a means of diagnosis. Currently differential diagnosis of Alzheimer's disease by clinicians is carried out by exclusion of other known forms of dementia namely multi infarct dementia (MID). Moreover, there are a number of other conditions (e.g depressive illness, acute confusional states) from which dementia needs to be differentiated (Huppert & Tym, 1986). The diagnosis of dementia and a judgement regarding the type of dementia is based largely on clinical findings supported by appropriate radiological and laboratory investigations (Mc Geer, 1986). Recently however, preliminary studies for a non invasive diagnostic test have been reported in the USA. It was observed that patients diagnosed with probable Alzheimer's disease or potentially at risk of developing it, exhibited a marked hypersensitivity to the pupil dilatory effects of the cholinergic antagonist tropicamide. This test awaits confirmation in larger trials (Scinto *et al.*, 1994).

Clinically, Alzheimer's disease is characterized by an insiduous onset which is gradually progressive. In the first stage, impairment of memory (short term memory) for recent events is usually the prominent feature. Disorientation especially in time and place frequently occurs (wandering). Fleeting and variable spells of impaired concentration, restlessness, anxiety and depression occur alongside other features of dementia. In the second stage all aspects of memory fail progressively, blunting of emotions and apathy begin to take over the mood and state. At this stage Parkinson-like symptoms have also been observed, psychotic syndrome with hallucinations and delusions may also be seen towards the latter end of this stage. In the third and final stage there is gross disturbance of all intellectual functions. There are marked neurological deficits and an increase in muscle tone appears with accompanying slow wide-based and unsteady gait. There is gross emotional disinhibition and the former personality becomes submerged - patients do not recognize relatives or even their own face in the mirror. Double incontinence is almost invariable at this stage. Progressive wasting is also seen to occur despite a voracious appetite (Huppert & Tym, 1986).

Neuropathology of Alzheimer's disease

Macroscopically, the classical feature associated with Alzheimer's disease is brain atrophy. In the cerebral cortex, the frontal and temporal lobes are strikingly affected with concomitant ventricular enlargement. These features are distinctive, but not necessarily diagnostic and in presenile cases brain atrophy may not even be pronounced (Perry, 1986). Post mortem studies have also shown that early hippocampal atrophy, is related to the Alzheimer's disease process and that it could be of potential predictive value (De Leon *et al.,* 1993).

The neuropathology of the disease at the microscopic level is complex. Post mortem Alzheimer histology is characterized by senile amyloid plaques, neurofibrillary tangles and neuronal loss. Neither plaques nor neurofibrillary tangles are unique to

Alzheimer's disease and are present to varying degrees in normal ageing (Perry, 1986) and other neurological diseases (e.g post encephalitic Parkinsonism)(Rowan, 1993). There has been much debate about the relative importance of these different pathological features and how they relate to each other. It has been shown by Braak *et al.,* (1993), that the distribution pattern of the amyloid deposits does not correlate to that of the neurofibrillary changes and that amyloid deposits are not necessarily a pre requisite for the development of neurofibrillary changes.

In both normal ageing and Alzheimer's disease, plaques are complex extracellular depositions which accumulate in the cerebral cortex as round or ovoid structures many times the size of individual neurons. There are three main components in a typical Alzheimer plaque - degenerating neurites (abnormally enlarged distal axons, dendrites and nerve terminals), glial processes and reactive astrocytes and a central amyloid core. The relative abundance of these differing components has been used to subclassify plaques and relate their features to plaque development and evolution. The more mature or classical senile plaque is distinguished by a central core composed of amyloid material surrounded by a peripheral rim of dystrophic neurites and reactive astrocytic cells (Perry, 1986). These dystrophic neurites surrounding the amyloid core of a mature senile plaque also contain paired helical filaments (PHF) - the main structural constituent of the neurofibrillary tangles (Hardy & Allsop, 1991). Microglia are also observed in the centre of the plaque (Rowan, 1993). Senile plaques are found to occur primarily in the neocortex and also in subcortical areas such as the hippocampus. It is believed that some factor or a lack of protection must make the cells in these areas more sensitive to the formation of senile plaques (Rowan, 1993). Heparin sulphate, other proteoglycans, inorganic compounds and other proteins such as α1 antichymotrypsin (an inflammation associated anti protease), apolipoprotein, acetylcholinesterase (AChE), complement, ubiquitin, and tyrosine hydroxylase have all been found closely associated with senile plaques (Mesulam & Geula, 1991; Rowan, 1993; Selkoe, 1991; Wilmot *et al.,* 1995).

Diffuse deposits of amyloid are also detectable in many brain regions of clinically unaffected individuals and it has been suggested that these deposits are precursors of the senile plaques (Rowan, 1993). Most diffuse plaques show no association with abnormal neurites or reactive glial cells and few or no amyloid fibrils. These are often the only form of amyloid plaque found in cases of Down syndrome below the age of 35 and are also found abundantly in the brains of ex boxers with dementia pugilistica (Hardy & Allsop, 1991; Roberts *et al.,* 1990). In Alzheimer's disease diffuse plaques are usually more numerous and widespread throughout the CNS than senile plaques (e.g often in the spinal chord and cerebellum) appearing as though they evolve into neuritic plaques in only specific brain regions (Hardy & Allsop, 1991).

A variety of neurotransmitters and neuropeptides have been shown to be present in plaque neurites. There is immunohistochemical evidence indicating that plaque neurites are derived from a variety of neuron types. Thus, of the classical neurotransmitter systems investigated, fluorescent studies have demonstrated catecholaminergic varicosities in plaques and both AChE histochemical reactivity and ChAT immunocytochemistry implicating cholinergic processes in aged human and monkey brain (Perry, 1986).

The neurofibrillary tangles first described by Alois Alzheimer are characterized by an abnormal filamentous accumulation of paired helical filaments in the cytoplasm of dying neurons. It is one of the major histological hallmarks of Alzheimer's disease and is essential for the pathological diagnosis of the disease. In contrast to plaque formation, tangles are not found to any significant extent in the neocortex of nondemented elderly brains, although in Alzheimer's disease tangles form to the greatest extent in the neocortex (Perry, 1986). Tangles are abundant in the pyramidal cells of the hippocampal formation and in the medial temporal cortex. They are also found in the association cortex and in certain subcortical nuclei in

particular the magnocellular nucleus of Meynert (cholinergic), the locus coeruleus (noradrenergic) and the raphe nucleus of the brain stem (serotonergic)(Price, 1986).

Abnormalities in the cholinergic neurotransmitter system in Alzheimer's disease

Neurons of the basal forebrain cholinergic system (i.e the medial septum, diagonal band of Broca and nucleus basalis of Meynert (nbM)), provide the major cholinergic innervation of the amygdala, hippocampus and neocortex (Price *et al.,* 1993). In Alzheimer's disease many nbM nerve cells have been found to develop neurofibrillary tangles and some neurons are reduced in size. Attempts have been made to link the deposition of β amyloid in Alzheimer's disease, with cholinergic involvement although the question of whether basal forebrain lesions induce plaques remains controversial. It has however been reported that increased synthesis of the β amyloid precursor protein occurs after nucleus basalis lesions and that the plaques found, are jointly reactive for the β amyloid peptide and acetylcholinesterase (Wallace *et al.,* 1991; Ulrich *et al.,* 1990).

There is a large body of evidence that abnormalities of the basal forebrain are related to memory loss and other symptoms of dementia (Perry *et al.,* 1988). Among the most characteristic changes in Alzheimer's disease is the early loss of basal forebrain cholinergic neurons in the nucleus basalis of Meynert - up to 90% in some cases (Giacobini, 1990). A loss of ACh content and the enzyme responsible for ACh synthesis - choline acetyltransferase (ChAT) has also been reported. Release of [^{3}H]ACh as measured in frontal cortex slices from human autopsy after short post mortem delay and also from biopsies have demonstrated a significant reduction (50-70%) in the evoked release of [^{3}H]ACh (Giacobini, 1990). Reports on the loss of ChAT from the brain cortex of Alzheimer patients resulting from the degeneration of basal forebrain cholinergic neurons have been amply confirmed and reductions of up to 90% are known to occur in the same areas known to contain high levels of

amyloid protein. Reductions in ChAT activity have also been shown to correlate well with the severity of cognitive deficits and density of plaques (Perry *et al.*, 1978). On the other hand it appears that normal ageing is not associated with such dramatic changes of ACh content and ChAT activity in the brain cortex or changes in the number of cholinergic neurons in the basal forebrain (Decker *et al.*, 1987; Henke & Lang, 1983). The derangement of cholinergic function seen in other forms of dementia rules out a primary involvement of ACh in Alzheimer's disease (Perry, 1994).

The great majority of cortical cholinesterase activity in the brain is expressed in the form of axonal and perikaryal AChE. However in Alzheimer's disease it has been found that AChE within normal cells and axons of the cerebral cortex is relatively low whereas intense AChE activity appears in the neuritic plaques, and neurofibrillary tangles. Electron microscopic histochemistry shows that this cholinesterase activity is ass ociated with amyloid deposits, abnormal neurites and paired helical filaments. Moreover in some areas of the Alzheimer brain, plaques and tangles appear to contain virtually all of the cortical AChE. Thus the cholinesterase positivity of cortical plaques and tangles is not an occasional event, but a widespread phenomenon and can therefore be considered to be one of the markers present in all the neuropathological structures associated with Alzheimer's disease (Mesulam & Geula, 1991).

Although the total number of muscarinic ACh receptors is not substantially changed in the brain cortex of patients with Alzheimer's disease, the number of M2 receptors has been shown to be diminished (Mash *et al.*, 1985). However, the largely postsynaptic M1 receptors appear to be increased and an increased level of M1 mRNA has also been isolated from the temporal cortex of patients suffering with Alzheimer's disease (Warpman *et al.*, 1993).

Numerous studies have also shown that the number of nicotinic ACh receptors is clearly decreased (30-50%) in post mortem Alzheimer brain tissue as well as in other forms of dementia as measured by the binding of radiolabelled ACh, nicotine and methylcarbamylcholine (Perry, 1994). It is thought that the M2 muscarinic and nicotinic ACh receptors that have been lost are those located presynaptically on the nerve terminals of basal forebrain cholinergic neurons. Furthermore, the loss of nicotinic receptors has been correlated to presynaptic changes including ChAT activity and loss of cells in the nbM which projects to the cortex (Giacobini, 1990).

Despite the preserved number of muscarinic receptors, a possible explanation for the poor effects seen with muscarinic agonists is thought to be due to disturbances in the receptor-mediated signal transduction in Alzheimer brain. It has been observed that the preservation of a neurotransmitter recognition site in a disease state is not necessarily an indication that the receptor is functionally intact - the functional integrity of a neurotransmiter receptor depends on its coupling to intact, associated signal transduction mechanisms. Thus, it has been suggested that these muscarinic receptors may be functionally impaired in Alzheimer's disease and is consistent with evidence of a degree of receptor uncoupling in the disease, which may account for some of the therapeutic inefficacy of the muscarinic agonists (Warpman, 1993).

Pearce and Potter (1991) however have shown that human cortical M1 muscarinic receptors labelled with 1nM [^{3}H]pirenzepine are not significantly changed in number, high and low affinity binding states, or GppNHp (a non hydrolysable analogue of GTP)-sensitive high affinity agonist binding, in Alzheimer's disease. The major postsynaptic muscarinic receptor associated with cerebral excitation belong to the M1 subtype, which is coupled to G proteins to activate phospholipase C. Further studies by these workers showed equal levels of M1 receptors in control and denervated rat hippocampi and also unchanged high and low affinity agonist binding

states, unchanged GppNHp-sensitive high affinity agonist binding and effectively normal agonist activated phospholipase C activity, 1 year after denervation. These observations are thought to support the idea that denervated M1 receptors remain fully capable of responding to agonists (Pearce & Potter, 1991).

Abnormalities in non cholinergic neurotransmitter systems

It has been shown that neurotransmitter systems other than the cholinergic system are also affected in Alzheimer's disease - namely the monoaminergic system in which 5-HT (5-hydroxytryptamine) and catecholamines act as neurotransmitters. Nerve bundles containing 5-HT originate in the raphe nuclei of the mid brain and innervate structures of the brain such as the striatum, limbic system and neocortex. Reduced cell counts and the presence of tangles in the raphe nuclei have been described in Alzheimer's disease and the maximum binding values have been reported to be 50% of the controls in the putamen and cingulate cortex (Regland, 1993). Reduced [^{3}H]5-HT binding has also been observed in post mortem samples of temporal cortex, but not in biopsy samples, suggesting that 5-HT receptor losses are most likely to be a feature of the latter stages of the disease. It has also been suggested that the loss of 5-HT receptors may be a reflection of the loss of cholinergic terminals on which they are thought to be localised (Bowen *et al.,* 1983).

It has been shown that noradrenergic deficits contribute to the pathogenesis of Alzheimer's disease in at least a subpopulation of patients, who suffer from considerable cortical noradrenergic depletion, decreased NA turnover, decreased levels of NA and neuron loss in the locus ceruleus (Adolfsson *et al.,* 1979; Bondareff, 1982). It has been observed that the severity of neuronal degeneration in the locus ceruleus is more evident in younger Alzheimer patients (Bondareff, 1994). A decrease in α1 and α2 and an increase in β1 and β2 adrenergic receptors has been reported (Kalaria *et al.,* 1989 & 1991). Nevertheless α2 and $5HT_{1A}$ sites in Alzheimer's disease have been shown to be capable of undergoing guanine

nucleotide-induced (cGMP) conversion from low to high affinity agonist states and are thought to be effectively coupled to G proteins (Cowburn *et al.,* 1993).

Evidence that noradrenergic dysfunction influences the cognitive deficits of Alzheimer patients is not well established, but a significant correlation between brain NA markers and performance on cognitive tests have been reported (Adolfsson *et al.,* 1979; Mann *et al.,* 1980). Evidence of a dysfunction of dopaminergic neurons has been less conclusive, although reports on the loss of DA in areas like the thalamus, hypothalamus, striatum and pons also exist (Regland, 1993).

The disturbances in neurotransmitter functions are obviously widespread in Alzheimer's disease not only affecting the cholinergic and monoaminergic systems but other neurotransmitter systems as well. Studies on the levels of the excitatory amino acid glutamate, the major neurotransmitter utilized by pyramidal cells of both the cerebral cortex and hippocampus, range from reduced glutamate levels, to no change at post mortem in Alzheimer patients (Dewar & Mc Culloch, 1994). Moreover in biopsy material, the K^+-stimulated release of glutamate, as well as the excitatory amino acid aspartate, has been shown to be unchanged in Alzheimer patients compared to controls (Smith *et al.,* 1983). However the biosynthetic enzyme of glutamate, glutaminase, was found to be depleted as were neurons stained with antibodies raised against this enzyme (Akiyama *et al.,* 1989). Depletion of [^{3}H]D-aspartate binding in cerebral cortex and hippocampus also suggest marked loss of glutaminergic terminals, although the use of this ligand as a specific marker of glutaminergic terminals has been questioned following the observation that lesions of glutamatergic pathways such as cortico-striatal and retino-collicular projections, failed to reduce [^{3}H]D-aspartate binding (cited in Dewar & Mc Culloch, 1994). Perhaps the most convincing evidence for the role of glutamate in Alzheimer's disease comes from the reduced number of pyramidal cell perikarya, which is reported to correlate with the severity of dementia (Palmer & Gershon, 1990).

A role for glutamate-mediated neurotoxicity has been supported by several lines of evidence. The demonstration that increased levels of glutamate in hippocampal cultures cause cytoskeletal changes similar to those observed in tangles, supports the notion that glutamate may be involved in excitotoxic processes in Alzheimer's disease (Mattson, 1990). However it is thought unlikely that excessive glutamate receptor activation is the primary event in the neurodegenerative process, although it is known that cells with compromised metabolic function exhibit increased susceptibility to N-methyl-*D*-aspartate receptor-mediated neurotoxicity (Novelli *et al.*, 1988).

Short intrinsic neurons in the cerebral cortex are thought to utilize GABA and a variety of neuropeptides such as neuropeptide Y, somatostatin and substance P. A loss of GABAergic innervation is indicated by reduced levels of GABA receptor markers (Simpson *et al.*, 1988) and reduced somatostatin immunoreactivity (Rossor *et al.*, 1980), which is co-localised with GABA in these non pyramidal neurons (Heridry *et al.*, 1984). Correlations have been noted between cortical somatostatin deficits and plaque and tangle densities. There is also evidence for some loss of neuropeptide Y, which also coexists with somatostatin in some cortical and hippocampal interneurons. Immunohistochemistry has also revealed a profound depletion of substance P reactive perikarya in a number of cortical regions (Dewar & Mc Culloch, 1994). There is also evidence for a loss of σ binding sites in the hippocampus in Alzheimer's disease which is consistent with pyramidal cell loss in the same region (Jansen *et al.*, 1993)

The discovery that specific growth (trophic) factors govern the survival, maintenance and response to injury, of key neurons led to an interest in determining the status of these growth factors and their receptors in Alzheimer's disease. Of special interest amongst these agents is nerve growth factor (NGF), which is now known to be associated with the basal forebrain cholinergic system (Wilcock & Dawbarn, 1994).

Research in the area of growth factors is two-fold; firstly to establish whether basal forebrain cholinergic pathology is due to disturbances in trophic factors and secondly, whether they may be a rationale for therapeutic approaches based on increasing the levels, synthesis and release of select growth factors such as nerve growth factor (Perry, 1994). However to date, no convincing evidence for a primary disturbance in nerve growth factor, or its receptor, or related factors, has been shown although the degeneration of nerve growth factor producing neurons such as those in the hippocampal pyramidal layer, suggest that some abnormality in the production of the respective growth factors is likely (Wilcock & Dawbarn, 1994).

Molecular pathology

The central amyloid core of senile plaques is composed of mainly highly insoluble β-pleated, unbranched proteinaceous fibrils which are unusually resistant to many solvents and proteolytic enzymes (Rowan, 1993). Eventually in 1984, a peptide consisting of about 39-42 amino acid residues was identified - now known as β/A4 peptide, β amyloid or amyloid β protein (AP)(Glenner & Wong, 1986). The source of β amyloid is now known to be β amyloid precursor protein (APP). This protein has at least three isoforms which contain the β amyloid sequence. They all have a single membrane spanning region, with a long extracellular N-terminal segment and a short intracellular C-terminal tail. The β amyloid sequence forms part of this transmembrane component (up to 14 amino acids) and extends to include some (approximately 28) extracellular amino acids. Two isoforms (APP_{751} and APP_{770}) contain inserts of Kunitz-type serine protease inhibitor domains, which have been found to be identical to protease nexin II. APP_{695} is the dominant form in the CNS (Rowan, 1993).

The endogenous APP gene is expressed in virtually every cell type both neuronal (astrocytes and microglia) as well as non neuronal (blood platelets, endothelial cells) tissues (Regland & Gottfries, 1992). The cellular source of β amyloid deposited in

Alzheimer's disease remains yet unclear, although the balance of opinion appears to be in favour of a neuronal origin (Murphy, 1992).

Neurofibrillary tangles, neurites of senile plaques and neuropil threads are composed of relatively insoluble paired helical filaments (Selkoe, 1991). Chemical and immunohistochemical studies of neurofibrillary tangles have identified ubiquitin, β amyloid and the microtubule associated protein - tau (Hardy & Allsop, 1991). Tau from Alzheimer brain is abnormally phosphorylated, consisting of three tau isoforms with molecular weights of 60, 64 and 68 (A68) Kd (Rowan, 1993). It has been claimed that this protein can only account for about 10% of the mass of paired helical filaments; the remainder is unknown (Hardy & Allsop, 1991).

The main role of tau, as a microtubule associated protein is thought to be to stabilise axonal microtubules in their polymerised state. Microtubules play an essential role in neurons in the maintenance of structure and also in providing the framework for orthograde and retrograde axonal transport. Evidence suggests that the abnormally phosphorylated forms of tau found in Alzheimer's disease, interacts abnormally with microtubules (Hanger *et al.,* 1991). Many antibodies have been shown to react with tau in the normal, as well as the phosporylated form of tau. AlZ50, a monoclonal antibody reacts specifically with the A68 isoform of tau and displays tangles, plaque neurites and neuropil threads very clearly (Osmand & Switzer, 1991).

It is still unclear how β amyloid is formed from APP. One of the major pathways for APP involves the cleavage of the molecule in the middle of the β amyloid sequence by proteolysis. Since this secreted form of APP contains only a part of the β amyloid sequence, neither this nor the remaining membrane bound fragment is likely to be the source of β amyloid in Alzheimer's disease (Rowan, 1993). However abnormal degradation of APP has been shown to result in the formation of fragments with intact β amyloid and its subsequent deposition. At present the identity of the

proteolytic enzymes involved in the normal and abnormal APP processing pathways are unknown, although it has been proposed that the endosomal-lysosomal pathway may be responsible for the abnormal degradation of APP (Hardy & Higgins, 1992).

It has been suggested that cholinesterases also have protease activity (Balasubramanian, 1984). Such proteolytic action could transform a circulating or neuronally formed APP, into the soluble β amyloid found within the plaques and could also contribute to the degenerative transformation of cytoskeletal proteins into the PHF of the neurofibrillary tangles. In this way the putative proteolytic activity of the plaque and tangle bound cholinesterase like substances would be expected to further contribute to abnormal protein processing (Mesulam & Geula, 1991).

Aetiology of Alzheimer's disease

Considerable progress has been made over the past decade in determining the identity of the protein and molecular events which are involved in the molecular pathology of Alzheimer's disease. The discovery that the gene coding for APP is located on chromosome 21, opened the way to establishing a genetic basis for the disease (Rosenberg, 1993). However it is now clear, that the underlying disease process does not have a single cause, but can be triggered by a number of genetic and/or environmental factors. Thus Alzheimer's disease is presently described as a heterogenous group of disorders; some being transmitted genetically and the majority being sporadic. Nevertheless the pathological sequence of events is believed to be the same in all cases.

The hypothesis that abnormal APP processing and the deposition of β amyloid, the main component of the neuritic plaque, is the central event in the aetiology of Alzheimer's disease has been widely debated and derives from several observations.

- Humans with Down syndrome (trisomy 21) are known to develop neuropathological features indistinguishable from those of Alzheimer's disease. They are known to develop typical β amyloid-bearing plaques and neurofibrillary tangles and a decline in cognitive functioning by their fourth decade. It is generally assumed that the invariant occurence of these β amyloid lesions, is related to the over expression of the APP gene caused by the extra copy of chromosome 21 (Masters *et al.,* 1985). The predictability with which persons with Downs syndrome develop the typical characteristics of Alzheimer's disease, has made it possible to investigate the time course of such degenerative events, as well as the chronological and pathological relationships between the the amyloid formation and neurofibrillary tangles.
- It has been found that APP processing is altered in Alzheimer patients having mutations in the chromosome containing the APP gene, located on chromosome 21. Recent identification of mutations in the APP gene on chromosome 21, in several different families in which Alzheimer's disease is inherited (familial), have provided further evidence for a causative role for β amyloid as well as a genetic basis for Alzheimer's disease (Ashall & Goate, 1994; Rosenberg, 1993). However it has been found that mutations in chromosome 21 are not present in all familial cases and evidence linking familial and sporadic Alzheimer's disease with other genes located on chromosomes 14 and 19, the gene for apolipoprotein E (apoE4) have also been reported (Ashall & Goate, 1994; Scott, 1993). Individuals that inherit 2 alleles of apoE4 are thought to be more likely (8 times) to have Alzheimer's disease compared to normal individuals. It appears that the genetic causes of Alzheimer's disease are themselves heterogenous.
- The possibilty that β amyloid-neurotoxicity may be a prominent cause of neuronal degeneration has been widely debated over the last few years. Initial studies in which *in vivo* hippocampal and cortical infusions of synthetic β amyloid or SDS-extracted β amyloid derived from Alzheimer brain were tested, suggested a neurotoxic effect in rat. Results of subsequent studies conflicted with these and it

is now thought that the discrepancies observed may be due to the aggregation status of the peptide and the concentration used (Rosenberg, 1993). The aggregated form is considered to be more toxic, although the mechanism by which it exerts toxicity remains unclear - apoptosis (where cell death occurs in response to internal metabolic cues that trigger a molecular programme of self-destruction) and destabilization of Ca^{2+} homeostasis have been proposed (Cotman *et al.,* 1993). ApoE4, a plasma lipoprotein, which is synthesized in the brain and has been shown to be localized in the senile plaques and neurofibrillary tangles in Alzheimer's disease, is thought to play a role in β amyloid aggregation (Wilmot *et al.,* 1995).

- Studies with transgenic mice, genetically programmed for neuronal over expression of human mutant APP, have shown that these mice express high levels of human mutant APP and also develop progressively some of the pathological hallmarks of Alzheimer's disease such as neuritic plaques, synaptic loss, astrocytosis and microgliosis (Games *et al.,* 1995).

Nevertheless, the majority of cases of Alzheimer's disease appear to be sporadic with only a small proportion of cases having a genetic basis. It has been proposed that sporadic cases may be either directly due to environmental factors, or have an inherited susceptibilty to environmental factors. Of the various environmental factors put forward as potential risk factors for Alzheimer's disease, one of the most significant is a previous history of head injury. Epidemiological studies have provided evidence for a link between head injury and the subsequent development of Alzheimer-type pathology - an apparent association in 2-20% of Alzheimer cases. Repeated blows to the head experienced by boxers are known to result in a dementing syndrome called dementia pugilistica, where widespread neurofibrillary tangle formation in the cortex has been observed (Roberts *et al.,* 1990). The presence of large numbers of diffuse β amyloid has also been observed, although no appreciable neuritic plaque formation has been seen to occur (Roberts *et al.,* 1990).

It has been suggested that the neuronal trauma and ischaemia which accompany head injury causes a cascade of molecular events resulting in APP over expression, which in certain susceptible individuals leads to mismetabolism and subsequent deposition of β amyloid protein (Gentleman *et al.*, 1993).

The involvement of aluminium (Al) in Alzheimer's disease has been implicated and is also considered to be a risk factor following a number of epidemiological studies. It has been observed that the incidence of Alzheimer's disease is significantly increased in areas where there are high residual levels of Al from the treatment of water supplies. Progressive cognitive decline has also been reported in miners chronically exposed to Al powder to prevent silicotic lung disease (Rifat & Eastwood, 1994). The brain content of Al is not found to be raised in neuropathologically assessed cases of severe Alzheimer's disease compared with pathology free controls, but focal deposits of aluminosilicate are present in the central core region of senile plaques and studies *in vitro* indicate that aluminosilicates can influence the fibrillary aggregation of β amyloid. It has been suggested that if Al contributes to the pathogenesis of sporadic Alzheimer's disease, it does so indirectly in susceptible individuals, possibly via effects on the biosynthesis or proteolytic processing of APP, or the processing of tau (Edwardson & Candy, 1992).

The herpes simplex virus type 1 (HSV1) which is known to be latent in the trigeminal ganglia of sufferers of cold sores has also been highly implicated in the pathogenesis of Alzheimer's disease. In population studies, HSV1 has also been shown to be prevalent in 85% by the age of 60. Furthermore the regions of the brain affected in herpes encephalitis are also those which show the main neuropathological features - plaques and neurofibrillary tangles in Alzheimer's disease (Itzhaki *et al.*, 1993).

A number of criticisms have been put forward concerning the amyloid hypothesis. First sporadic (non familial) cases of Alzheimer's disease which are undoubtedly the

majority, are unlikely to be explained by an over expression of the APP gene or a mutational defect. Moreover recent genetic studies have clearly shown that hereditary Alzheimer's disease has several aetiologies - in some cases defined mutations in the β amyloid gene on chromosome 21 have been shown to occur, whereas in other cases such mutations are absent (Scott, 1993; Ashall & Goate, 1994). Also recent transgenic mouse models of Alzheimer's disease which have been able to express high levels of human mutant APP do not develop neurofibrillary tangles, characteristic of Alzheimer's disease (Duff & Hardy, 1995). Furthermore amyloid plaques which are common in the cortex in normal ageing and are present in high density in cognitively normal elderly patients are accompanied by little neuronal alteration or mental dysfunction, possibly because the β amyloid deposition is not the central event in the pathogenesis of Alzheimer's disease. An alternative hypothesis suggests a protective role for APP rather than a toxic one, whereby Alzheimer's disease is most likely to develop when APP is insufficient either because of a genetic effect, or because of intense exposure to causal factors in the environment which produces APP which is both protective and amyloidogenic (Regland & Gottfries, 1992).

Alzheimer's disease models

Whereas the classical neuropathological features - the senile plaques and the neurofibrillary tangles provide the keystone for post mortem diagnosis of Alzheimer's disease, changes in the functional activity of a wide variety of identified neurotransmitters have also been established as discussed previously. So far it has proved difficult to ascertain which neuropathological and neurochemical changes are primary in the aetiology and development of the disease, or which might be responsible for the different psychopathological features.

The discovery that Alzheimer's disease is associated with a loss of basal cholinergic neurons, as well as the correlation of cholinergic abnormalities with the severity of

memory loss and other symptoms of dementia (Perry *et al.*, 1978), stimulated research on the role of cholinergic mechanisms in learning and memory with a view to developing more efficient cholinergic pharmacotherapy.

The importance of central cholinergic systems in learning, memory and cognition has been suggested from the pharmacological studies accumulated from both animals and humans. Many studies, for example have demonstrated that antimuscarinic agents such as atropine and scopolamine, can disrupt both the acquisition and performance of a variety of learned behaviours. In rodents, primates and man there have been numerous reports that scopolamine produces memory impairments similar to that seen in Alzheimer's disease (Dunnett, 1994). However subsequent studies have shown that the memory deficits produced by scopolamine, produce a non specific disruption of performance which are substantially different to those seen in Alzheimer's disease (Fibiger, 1991). To some extent these differences, are not surprising because muscarinic antagonists produce an acute, short-lasting blockade of cholinergic receptors that are primarily postsynaptic and anatomically non specific, while Alzheimer's disease is a chronic, irreversible disorder which involves anatomically specific changes in presynaptic cholinergic function.

An alternative to studying the role of cholinergic systems in learning and memory has been to lesion selected populations of cholinergic neurons. The identification of an affected population of cortically projecting cholinergic neurons that are degenerated in dementia, has stimulated researchers to reproduce this deficit in animals by experimental lesions using a variety of techniques - surgical, electrolytic, neurotoxin methods. However problems have arisen concerning the specificity of the lesions produced, particularly those that are surgically induced. Nevertheless neurotoxins such as cholinotoxins and excitotoxins which are used regularly in lesioning studies and have often demonstrated improved selectivity (Cutler *et al.*, 1994).

Administered in appropriate concentrations, neurotoxins are capable of destroying cell bodies at the site of injection in the basal forebrain. In both rodents and primates, lesions of the nucleus basalis and or medial septum produce deficits in learning, although the nature of the deficit is not completely the same for each area. Impairment produced by these lesions can be reversed using a cholinesterase inhibitor or a directly acting agonists (Dunnett, 1994).

Of the cholinotoxins available, the strongest claims have been made for ethylcholine mustard aziridinium (AF64A or ECMA), a nitrogen mustard analog of choline which is an irreversible choline uptake inhibitor. Lesions of the nucleus basalis of Meynert by AF64A have shown significant decreases in hippocampal ChAT activity, as well as presynaptic cholinergic dysfunction similar to Alzheimer's disease and also marked and long lasting impairments in memory and other cognitive behaviour (Cutler *et al.,* 1994). However the neurochemical specificity of AF64A appears to depend on the route of administration - intraventricular infusion being the favoured route (Fisher *et al.,* 1982; Walsh *et al.,* 1984) rather than intraparenchymal infusion, which appears to induce extensive nonspecific, gross cellular toxicity (Allen *et al.,* 1988) thus limiting the usefulness AF64A.

Lesions of terminal regions have also been simulated with the direct injection of hemicholinium (HC-3), a reversible choline uptake blocker, into the hippocampus or into the ventricles. This produces an impairment in memory on both passive avoidance and spatial tasks, although this effect is not consistently found in all studies (Colhoun & Rylett, 1986).

Many groups have used excitatory amino acid neurotoxins such as kainic acid or ibotenic acid, to lesion the nucleus basalis of magnocellularis which provides cholinergic projections to the neocortex. However it has been shown that the effects of such lesions indicate a disturbance of some form of conditional discrimination

learning, or attentional process, rather than mnemonic basis for learning (Dunnett *et al.,* 1991). Furthermore detailed anatomical analyses of the effects of ibotenic acid infusion into the basal forebrain have indicated that the magnitude of the behavioural deficits correlated not so much with the degree of reduction in cortical ChAT activity, as with the magnitude of the lesions, in terms of general cellular destruction in the dorsal and ventral globus pallidus (Everitt *et al.,* 1987), rendering the cholinergic interpretation of the behavioural deficit somewhat problematic.

Alternative toxins such as quisqualic acid and the excitotoxic amino acid receptor agonist α amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) have been found to be effective in destroying the nucleus basalis of magnocellularis cell bodies leading to reductions in neocortical ChAT activity in excess of 70%, while sparing most noncholinergic neurons (cited in Dunnett *et al.,* 1991). However with AMPA lesions there were no effects on acquisition or retention in the spatial water maze, thus making the interpretation of excitotoxin lesions difficult (Page *et al.,* 1991).

Thus the evidence supporting the role of cholinergic mechanisms in learning and memory is vast. Recently however, the role of NMDA receptors in memory has been investigated following the proposal that long-term potentiation (LTP) may be a mechanism of memory. The phenomenon of LTP, an enduring change in synaptic efficiency, has been extensively demonstrated in the hippocampus and also described in the amygdala and cortex (Brown et al., 1990). Lesion data suggest that memories are not stored either in the hippocampus or in the amygdala, since animals with bilateral ablation of the hippocampus and amygdala do not lose memories but become incapable of learning new ones (cited in Izguierdo, 1991). Evidence to implicate glutamatergic neurotransmission both in LTP and in learning and memory come from the observations that NMDA antagonists such as MK-801 (dizocilpine), AP5 (2-amino-5-phosphonopentanoic acid) and AP7 (2-amino-7-phosphonoheptanoic acid), prevent the development of LTP and impair the

performance of experimental animals in tasks that require learning and memory (Izguierdo, 1991). Thus glutamatergic dysfunction may play a significant role in the memory impairments that characterize Alzheimer's disease.

An immunotoxic approach has been recently described using the nerve growth factor (NGF) receptors located on the cholinergic neurons in the basal forebrain. The toxin saporin has been conjugated to NGF receptor antibodies, in order to target its toxic action and has been shown to be effective in destructing the septal and nucleus basalis of magnocellularis cholinergic neurons. This novel approach awaits re evaluation. Thus it is evident that the choice of neurotoxin is important, as some of the deficits observed may not be related to decreases in ACh (Wiley *et al.,* 1991).

Behavioural models and behavioural testing have been a successful approach in developing anxiolytics, anti-parkinsons and also neuroleptic drugs, although they have failed to screen and predict clinical efficacy of potential anti Alzheimer drugs to a similar extent for many reasons. One of the major reasons for this failure is likely to be the difficulty of addressing the complexicity of symptoms present in the Alzheimer patient. Disturbances of the higher cortical function characteristic of cognitive impairment in Alzheimer's disease such as aphasia, apraxia and agnosia are difficult to study in animals. A further complication is that the irreversible progression seen in Alzheimer's disease cannot be mimicked in experimental lesions. However most of the cholinomimetic drugs being developed and used in the treatment of Alzheimer's disease today, have been screened and tested using many or most of the animal models described above and will continue to do so until animal models more relevant to the Alzheimer pathology as well as the symptoms are developed.

Recently however, two novel animal models have been developed which display many of the neuropathological characteristics of Alzheimer's disease. These are the transgenic mouse model and the animal model of Down's syndrome - the trisomy 16

mouse (Cutler *et al.*, 1994). The transgenic mouse model uses the human APP gene encoding mutations associated with familial Alzheimer's disease and via transgenesis introduces extra copies into newly fertilized mouse eggs. Over expression of the APP gene in these transgenic mice has been reported to show neuropathology strikingly similar to that of Alzheimer's disease, although there is no evidence of neurofibrillary tangles (Duff & Hardy, 1995). However the reproducibilty of this model has been questioned and as these mice have not yet been assessed for cognitive dysfunction, it remains to be seen whether the mice will be useful for testing therapeutic compounds.

As mentioned previously Down's syndrome subjects develop Alzheimer-like characteristics such as basal forebrain degeneration, neuritic plaques and neurofibrillary tangles. Some researchers have investigated the possibility of an animal model of Downs syndrome - the trisomy 16 mouse. Because trisomy fetuses die in utero or shortly after birth, trisomy 16 basal forebrain transplants are used to study the molecular pathogenesis of neuronal deficits in both Alzheimer's disease and Downs syndrome. Immunocytochemical staining techniques have suggested the presence of proteins associated with Alzheimer's disease in hippocampal grafts of trisomy 16 mice, thus suggesting another possible model of Alzheimer neuropathology (Richards *et al.*, 1991).

Therapy

Although pathological events such as amyloid plaques and tangle formation are currently viewed as the primary target in the development of therapeutic strategies, the need for further treatment of the neuronal damage and related clinical symptoms has also been recognized. Irrespective of the aetiology, the strong evidence correlating the loss of cholinergic markers with memory loss and cognitive dysfunction in Alzheimer's disease has suggested cholinergic therapy, as a means of reducing the symptoms and improving cognitive function. Although there is no

incontrovertible evidence that the cholinergic deficit in Alzheimer's disease is the central specific cause of cognitive losses, there has been adequate support for a role for cholinergic activity in cognition and for this reason, considerable effort has been turned to the development of therapies that compensate the cholinergic loss in Alzheimer patients. Thus even with the theoretical advent of antiamyloidogenic agents, other drugs aimed at improving the transmitter deficits are also thought to be a likely and necessary adjunct.

A variety of other substances such as the nootropics, NMDA antagonists and benzodiazepines have also been shown to enhance learning and memory functions (Schindler, 1992; Izquierdo, 1991). Therapeutic agents have also been aimed at restoring NMDA receptor function by activating its glycine modulatory site. Milacemide, which is converted to glycine in the CNS, has been shown to facilitate memory retrieval in young and aged healthy adults (Dewar & Mc Culloch, 1994).

The strategies aimed at enhancing cholinergic activity which have been clinically explored so far are - the use of the precursor (choline), cholinesterase inhibitors (ChE-inhibitors) and cholinoceptor agonists. None of these approaches however, has achieved the degree of benefit to Alzheimer patients anywhere near that which is achieved with drugs like L-dopa for the treatment of Parkinson's disease, possibly because the degenerating nerve cells include cholinergic as well as non cholinergic. The two most promising approaches so far have been cholinesterase inhibition and cholinoceptor stimulation. K^+ channel blockers such as the aminopyridines (4-aminopyridine and 3,4-di aminopyridine) which are known to augment cholinergic transmission (both basal and depolarization induced ACh release), have also been tested. The more recently investigated K^+ channel blocker linopiridine (Du 996) is thought to be more favourable as it has been shown to specifically enhance K^+ evoked ACh release (De Souza, 1993).

• Precursor loading - An increase in ACh synthesis was attempted by means of providing an excess of the precursors involved. At first choline itself was used, but it was found that in a high proportion of Alzheimer patients it was broken down by intestinal bacteria to trimethylamine, which smells of rotten fish. Choline was therefore substituted by phosphotidylcholine (lecithin), which when given in highly pure doses for 4 months showed favourable results in one control trial, among a subgroup of older patients, whose plasma choline levels rose to twice the baseline level (Little *et al.,* 1985). However the drug group as a whole showed no improvement in memory and moreover other workers were unable to replicate this improvement, using high purity lecithin albeit in a younger group of patients (Levy, 1994).

• Cholinoceptor agonists - Five muscarinic receptor agonists have been tested in Alzheimer's disease; these are RS-86, pilocarpine, oxotremorine, arecoline and bethanechol. The most widely reported in this category is RS-86, which has shown some improvements in a few patients, but has now been discontinued due to its ineffectiveness and high toxicity. Arecoline has also been investigated by introducing it into the cerebral ventricle (intrathecally), although the invasive nature of this procedure has resulted in numerous serious unwanted effects such as meningitis and severe extrapyramidal symptoms (Spiegal, 1989). These first generation agonists were unsuccessful due to their poor oral bioavailability, short duration of action and poor selectivity for postsynaptic (M1) receptors. Moreover the peripheral side effects associated with non selectivity, also make these drugs dose limiting (Giacobini, 1993).

Recent advances in the development of muscarinic receptor agonists have been aimed at specific subtypes, following the identification of at least five muscarinic receptor subtypes (M1-M5) in the brain. As mentioned above, most of the muscarinic agonists which have been evaluated in Alzheimer's disease show adverse peripheral

side effects and are either non selective (such as RS-86 which is a mixed M1/M2 agonist), or M2>M1 selective (Fisher *et al.,* 1991). These may also activate M2 autoreceptors resulting in a decrease of ACh release. Therefore M1 agonist, M2 antagonist or M1 agonist/M2 antagonist compounds have been suggested to be likely prospects for the treatment of Alzheimer's disease (Fisher *et al.,* 1991; Potter, 1987), although M2 antagonists would be expected to have major peripheral side effects on cardiac and smooth muscles.

However, the most important fact considered to be relevant to the development of a specific muscarinic agent for treatment of Alzheimer's disease is that most of the mRNA in the cerebral cortex and hippocampus is for M1 and M3 receptors. Therefore activation in the cerebral cortex and hippocampus in Alzheimer's disease is most likely to be enhanced with selective agonists for for these receptors. Since M1 receptors (which cause excitation in the cortex and hippocampus) are so far known to exist only in the brain, whereas M3 receptors are also prevalent peripherally, the best single choice for further development is considered to be an M1 agonist (Potter, 1992).

The choice of an M1 specific agonist is further supported by reports that M1 receptors are unchanged or increased in Alzheimer's disease. Furthermore experimental lesions of the fimbria/fornix (which produces total cholinergic denervation) were also shown to cause no loss of M1 receptors in the rat hippocampus, 1 year after lesioning (Pearce & Potter, 1991). The same study also showed no change in the high affinity binding of M1 agonists, or the ability of these agonists to activate phosphoinositide hydrolysis (Pearce & Potter, 1991).

Recently structurally novel muscarinic agonists have been synthesized with the anticipation of overcoming limited oral activity, short duration of action and peripheral side effects seen with the classical agonists. Included in this "new generation

agonists" are, arecoline oximes, oxadiazoles, compounds of the AF series, spiromides related to RS-86, pilocarpine and oxotremorine analogues (Schwarz *et al.,* 1991). AF102B, a rigid analog of ACh has been shown to have a relatively high selectivity for M1 receptors (Fisher *et al.,* 1991). It is reported to improve learning and memory defects in animal models and is thought to be a promising drug for the treatment of Alzheimer's disease (Fisher *et al.,* 1991). Release of ACh in rat brain by muscarinic agonists has also been reported - the prototype of such compounds being BM5, an oxotremorine analogue which is reported as having presynaptic antagonistic and postsynaptic agonistic properties (Nordström *et al.,* 1983). SDZ ENS 163, a pilocarpine derivative, is also thought to be highly specific for the M1 muscarinic receptors and is also known for its ACh releasing properties, which have been attributed to its M2 antagonist effect, similar to BM5 (Enz *et al.,* 1992). Despite its pronounced M2 antagonist activity, *in vivo* studies have shown that it has no effect on blood pressure and heart rate after intraperitoneal administration of this compound - clinical studies continue. More recently xanomelonine, also a specific M1 agonist, is currently in Phase II of clinical development for mid moderate Alzheimer's disease patients (Bodick *et al.,* 1994). *In vivo* studies have shown that xanomelonine does not produce hypothermia, salivation or tremor at doses up to 100mg/kg, although dose-dependent increases in heart rate were observed (Bodick *et al.,* 1994).

The development of nicotinic agonists for the treatment of Alzheimer's disease has not been pursued as actively, possibly due to the rapid desensitization of nicotinic receptors. An interesting aspect of the nicotinic receptor is that unlike most transmitter receptors, it is upregulated by its agonist (thought to be due to its rapid desensitization) and this increase in receptor number is evident for example in the brain of smokers, which may reflect enhanced function. Thus the observation that smoking protects against Alzheimer's disease could be explained on the basis of nicotine off setting the disease-related receptor decrement (Wonnacott, 1990).

A broader class of agents called cholinergic channel activators have been used to describe agents which are nicotinic receptor agonists as well as allosteric modulators. These agents are thought to interact selectively with neuronal nicotinic receptor subtypes at a site distinct from the ACh binding site (an allosteric site), which is not subject to the same desensitization mechanisms. ABT-418 ((S)-3-methyl-5-(1-methyl-2-pyrrolidinyl)isoxazole), is a prototype of this class which selectively activates neuronal nicotinic receptors, without eliciting the dose limiting side effects typically observed with (-)nicotine (Arneric *et al.,* 1994).

• Cholinesterase inhibitors - these are aimed at increasing the duration of released ACh, which in turn prolongs stimulation of post synaptic receptors which are largely intact in Alzheimer's disease. Eserine (physostigmine), a reversible carbamate cholinesterase inhibitor was the first ChE-inhibitor used in the treatment of Alzheimer's disease and has also been used in the treatment of atropine poisoning and also in ophthalmologic practices as a mi otic agent. Several investigators have reported improvement only with moderate doses of intravenous (i.v) eserine and failure to induce improvement at both high and low doses, giving rise to an inverted U-shaped dose response curve of improvement. The bulk of evidence suggests that the administration of i.v eserine can transiently improve memory and other psychological functions to a small extent. Oral eserine, despite being poorly and irregularly absorbed by the gastrointestinal tract also gives significant improvement when given repeatedly every 2-3 hours. This improvment has been shown to be restricted to particular tasks and only a subgroup of patients have been shown to be "clinically significant". The degree of dementia and the dose are thought to be major determinants of response. Doses of 9-15mg eserine produce peak plasma levels ranging from 0.5-2.4ng/ml; the plasma and brain half life being short ~ 15-30 min (Thal, 1991).

The limitations of eserine as a treatment for Alzheimer's disease is its short acting effect and also its side effects - the most common being nausea, vomitting, diarrhoea and sweating. Clinical studies of a novel form of slow releasing eserine derivative called eptastigmine (heptyl-physostigmine or MF-201), have shown that it has a longer duration of action and lower hepatotoxicity compared to other ChE-inhibitors (eserine and tacrine)(Imbimbo & Lucchelli, 1994), although high doses have revealed neutropenia in certain studies (Canal et al., 1994).

In 1986, Summers' *et al.* reported marked therapeutic improvement with a long lasting cholinesterase inhibitor called tetrahydroaminoacridine (THA/tacrine), in 17 patients in a double blind crossover trial. These workers also claimed that tacrine not only caused an improvement in test scores, but additionally caused worthwhile changes in activities of daily living. However Summers trial was subject to severe criticism on methodological grounds, although major efforts to replicate the study in better designed trials still continued. Promising results were reported in open studies (Gauthier *et al.* 1989), followed by disappointing results from a double blind placebo controlled trial (Gauthier, 1990).

Two other reports however still stand as obtaining positive benefits. Tacrine was shown to produce statistically significant and clinically important improvement compared to placebo in the performance of patients with Alzheimer's disease in the studies performed by Warner Lambert-Parke Davis in the US and independently by Eagger *et al.,* in the UK (Eagger *et al.,* 1991, Wallace, 1992). However these studies showed that while improvement correlated with plasma tacrine levels, there were also significant unwanted side effects. The most commonly seen side effects of tacrine are nausea, vomitting, diarrhoea, sweating and hepatotoxicity. Tacrine was discontinued for a while because of these side effects, namely an elevation of liver enzymes and hepatotoxicity but was recently introduced in Europe and reintroduced

in the US following favourable reports from two independent groups (Farlow *et al.*, 1992; Knapp *et al.*, 1994).

The mechanism of action of tacrine is varied as well as complex and it is thought that the improvement in cognitive function may not be entirely due to inhibition of ChE. Tacrine is a reversible inhibitor of ChEs, more potent on butyryl-cholinesterase than on AChE and its binding site is thought to be a hydrophobic area, close to the active site (Freeman & Dawson, 1991). It is not as potent as eserine, but has a longer half-life of 2.5h (Åhlin *et al.,* 1991), which adds to its therapeutic efficacy. The therapeutic plasma concentrations range from 0.2-0.6μM (Baldwin *et al.,* 1991; Summers *et al.,* 1986).

A second possible mechanism of action may be a direct effect on cholinoceptors. Tacrine has also been found to interact with muscarinic and nicotinic receptors (De Sarno *et al.,* 1989; Nilsson *et al.,* 1987) and is also reported to inhibit carbachol stimulated phosphoinositide (PI) hydrolysis at concentrations (Freeman & Dawson, 1991) which agree closely with its ability to displace muscarinic ligand binding. Tacrine has also been reported to release DA from the striatum and 5-HT and NA from the cortex (Baldwin *et al.,* 1991; Drukarch *et al.,* 1988; Robinson *et al.,* 1989) and also to inhibit the enzyme responsible for the breakdown of the monoamines - monoamine oxidase (MAO)(Nordberg *et al.,* 1989; Adem, 1992). It has been speculated that these effects may also play a significant role in some of the improvements seen in Alzheimer's disease. Several studies have also shown that tacrine directly affects Na^+, K^+ and Ca^{2+} channels (Marsh *et al.,* 1990; Halliwell & Grove, 1989; Griffith & Sim, 1990; Osterrieder, 1987; Drukarch *et al.,* 1987; Chaki *et al.,* 1991).

A number of other cholinesterase inhibitors are currently under investigation. These include velnacrine (a hydroxyl derivative of tacrine), galanthamine a cholinesterase

inhibitor extracted from the caucasian snowdrop, the antimalarial metriphonate, heptylphysostigmine (now withdrawn) and also the novel piperidine based cholinesterase inhibitor E 2020 (Levy, 1994). E 2020 which is chemically unique, has been shown to be an effective and relatively specific inhibitor of AChE, with dose-dependent activity that has greater selectivity for AChE and a longer duration of inhibitory action than either eserine or tacrine. Moreover toxicology studies as well as initial clinical studies have indicated that E 2020 is devoid of toxicity (Rogers *et al.*, 1991).

The use of ChE-inhibitors is thought to be the more favourable cholinomimetic approach and probably the most promising so far. Whereas agonists stimulate cholinoceptors on cholinergic neurons which improve the modulatory effect of ACh on these cells, ChE-inhibitors aim to preserve and enhance the physiological action of the neurotransmitter by increasing its level close to normal concentrations in the surviving neurons (Giacobini, 1993).

The balance of opinion is that the ChE-inhibitors tacrine and eserine improve cognitive function for a variable length of time in at least a proportion of patients with Alzheimer's disease. However attempts are being directed at widening the therapeutic scope and developing a more advantageous risk-benefit profile with fewer peripheral side effects and a lower likelihood of hepatotoxicity.

ChE-inhibitors are also important in view of the fact that AChE may play an important role in the pathogenesis of Alzheimer's disease. As mentioned previously, amyloid plaques and tangles are known to have high AChE activity. It is thought that AChE may have a direct or indirect proteolytic mechanism, thus participating in the formation of plaques and tangles by means of amyloid processing and accumulation. Histochemical studies have shown that tacrine and eserine not only inhibit the AChE of normal axons and perikarya, but also the AChE of plaques and tangles (Mesulam

& Geula, 1991). However biochemical studies have shown that AChE extracted from senile plaques are less sensitive to the ChE-inhibitors tacrine, eserine and E-2020. It was suggested that the intrinsic properties of AChE present in senile plaques might be altered (due to a conformational change in structure) compared to normal brain AChE, or that the accessibilty of the ChE-inhibitors is impeded in plaques (Nakamura *et al.,* 1993). However it must be noted that the extraction of AChE in this study with Triton X-100 and other salts may have contributed to the altered properties of the enzyme.

It is suggested that the correlations between the cognitive deficits, typical pathological changes and neurochemical markers all support the notion that loss of cortical neurons, synapses and decrease of ChAT are more supportive of pathogenetic mechanisms, than concentration of plaques and amyloid or density of tangles (Giacobini, 1993). However the success of the ChE-inhibitors in the treatment of Alzheimer's disease has not been that remarkable and does not support these claims. It has been postulated that steady state extensive inhibition of ChE, (measurable in plasma BuChE and CNS ACh levels) but not drug concentration, relates to a favourable clinical reponse (Becker & Giacobini, 1988). Clinical observations are apparently in agreement and it has been argued according to this hypothesis that the adverse and toxic effects of ChE-inhibitors are unlikely to be related to their plasma levels of ChE inhibition but to the intrinsic properties of the compound (Giacobini, 1993). Futhermore it has been reported by Hallak & Giacobini (1989), that although a direct correlation between the inhibition of AChE and extracellular ACh levels have been observed with most ChE-inhibitors, several discrepancies are also noted, where this relationship is not apparent (in rat brain). It was concluded that the change in AChE activity in the CNS is not the only factor regulating extracellular ACh concentration - ACh release is also regulated by cholinergic autoreceptors as well as by other neurotransmitters (Giacobini, 1993). De Sarno *et al.,* (1989) showed that tacrine, eserine and eptastigmine are also ligands

for both muscarinic and nicotinic cholinoceptors. Thus receptor interaction (as well as ChE inhibition) may be an important determinant of ACh levels, since it is well established that release of ACh is modulated by both muscarinic and nicotinic receptors.

Aims and objectives

Tacrine and eserine are the most clinically investigated ChE-inhibitors in Alzheimer's disease (Becker & Giacobini, 1991), even though eserine (oral or i.v) or tacrine (oral) in humans cause a maximum inhibition of 25-30%, at doses minimising side effects (Giacobini *et al.,* 1991). It is suggested that the encouraging clinical effects reported with the administration of tacrine and eserine might also be a reflection of inhibition of plaque and tangle AChE activity (Mesulam & Geula, 1991) and also an action at the cholinoceptors and other receptors. From the present account it is clear that in addition to its potency for inhibiting ChE, measurements of brain ACh levels *in vivo* and *in vitro* are also considered important in determining the overall efficacy of a ChE-inhibitor as a cholinomimetic. For this reason the present work investigates the mechanisms by which ChE-inhibitors affect the availability of ACh by studying the effect of tacrine, eserine, neostigmine and edrophonium on the release of K^+-evoked [^{3}H]ACh from rat hippocampal slices, taking into consideration possible receptor interactions.

CHAPTER 2

General Methods and Materials

INTRODUCTION

A variety of methods have been developed for the measurement of *in vivo* and *in vitro* neurotransmitter release in the CNS. *In vivo* methods of the past have included the cortical cup method used for superficial areas, the push pull cannula for deeper lying structures and the ventricular perfusion technique. Recently however, it appears that these techniques have largely been replaced by *in vivo* microdialysis, which has the advantage of measuring direct and regional transmitter release in the conscious animal.

Static and dynamic methods are used in the measurement of neurotransmiter release *in vitro*. The static method examines release by incubating the tissue preparation in a fixed volume of an appropriate medium for a specified period of time, after which the preparation is either rapidly cooled, filtered or centrifuged. The supernatant is then assayed for the neurotransmitter under investigation. The major limitations to this method are that released transmitter may be re-accumulated by the tissue, or build up in extracellular concentration so as to exaggerate feedback or feedforward mechanisms. Furthermore rapid cooling may shock the tissue resulting in artifactual release, or may terminate release completely. Another drawback with this method is that it is not suitable for investigating time phenomena. These problems decrease the sensitivity to small changes in release produced by a particular treatment (Minnema & Michaelson, 1985).

The dynamic approach uses superfused synaptosome, slice or prism preparations and overcomes many of the limitations of the above mentioned method. Continuous superfusion when optimized, obviates problems associated with the accumulation of neurotransmitters and permits the establishment of a baseline rate of release for each tissue sample, from which effects of treatment can be compared (Minnema & Michaelson, 1985).

Superfusion of brain slices or prisms has been used to examine the release of a variety of neurotransmitters - eg. catecholamines, GABA, 5-HT and ACh. Many studies investigating ACh release, have used the radiochemical method which measures the release of radiolabel after preloading with [^{3}H]choline (or [^{14}C]choline). Such isotopic methods of estimating ACh release do not necessarily reflect the actual amount released, since unlabelled ACh pre-existing in the tissue may also contribute (Beani *et al.,* 1984; Richter, 1976). It has also been reported that the synthesis of labelled ACh accounts for only 50-60% of the total amount of ACh synthesized during preloading and before release (Molenaar *et al.,* 1973). Thus the radiolabel released is inevitably diluted by the concominant release of unlabelled ACh.

The alternative method does not preload the tissue with a radiolabel, but simply measures the unlabelled ACh released. Beani *et al.* (1984), compared these methods using electrically stimulated cortical slices. They concluded that the method based on [^{3}H]choline preloading allowed drug-induced inhibition of release to be detected more readily than potentiation. However the radiochemical method used throughout the work reported in this thesis was clearly capable of detecting the potentiation as well as inhibition of release - as will be seen in the following chapters.

Although these methods have been well established they require the use of ChE-inhibitors to overcome the difficulty of measuring small amounts ACh. The use of ChE-inhibitors however, may lead to unrequired pharmacological effects such the activation of autoreceptors that modulate ACh release. Recently Umeda and Sumi (1990), introduced a highly sensitive method for assaying unlabelled ACh release, which does not require the use of ChE-inhibitors. This static method measures basal and K^{+}-evoked release of unlabelled ACh, albeit in the presence of hemicholinium (HC-3). ACh released was hydrolyzed and phosphorylated with [^{32}P]ATP. Significantly, potentiations of [^{3}H]ACh release described in this present thesis were

no less readily detectable than those of unlabelled ACh release reported by Umeda and Sumi (1990) (Chapter 3).

Further studies by Hadhazy and Szerb (1977) have compared the release of [^{3}H]ACh evoked by electrical stimulation and 25 mM K^+. It was observed that high K^+ caused a greater and a more prolonged release of the label than did electrical stimulation. It is thought that one of the reasons for this might be that high K^+ concentrations would affect all terminals and cause release from a larger pool than would electrical stimulation unless stimulated maximally (Hadhazy & Szerb, 1977). It was also observed that the quantity of [^{3}H]ACh which could be released by K^+ was larger (about double in the case of cortical and hippocampal and triple in striatal slices) than that which could be released by electrical stimulation. Although it is possible that K^+ stimulation might additionally release ACh from cell bodies and dendrites while electrical stimulation may not, it is unlikely that this would cause a significant contribution to the total amount of ACh released from the axon nerve terminal.

A variety of tissue preparations are used for the study of neurotransmitter release in the mammalian nervous system. Amongst these preparations, the hierarchy of complexity is from ganglia *in vitro*, tissue slices or prisms, and finally to synaptosomes. The most commonly used tissue preparations for the measurement of neurotransmitter release *in vitro* are synaptosomes and slices or prisms.

Synaptosomes are thought to have the advantage of synaptic accessibility in that they avoid the confounding influences of non neural tissue, membrane barriers and neuronal loops. However factors limiting their use are transmitter heterogeneity, contamination with gliosomes and lack of integrity and intactness (Minnema & Michaelson, 1985). In contrast to synaptosomes, slice or prism preparations may retain neuronal cell bodies and axonal processes. This may be useful when it is

required to determine whether exogenous ligands modify release by an action on the cell body or on the nerve terminals. However the hippocampus which receives more than 85% of its cholinergic innervation extrinsically from the medial septum, the slice or prism preparation would be expected to retain these cholinergic nerve terminals intact making it useful in the investigation of the presynaptic modulation of ACh release.

Slices or prisms have been used widely in the CNS, in the study of ACh - its synthesis and release, and the regulation and regional differences of these processes including interactions with other neurotransmitters. The nomenclature for prisms and slices is quite often interchanged in the literature. Prisms are defined as tissue cut by means of a mechanical tissue chopper at fixed intervals in two directions at a defined angle. Slices are defined as tissue cut either mechanically in single or consecutive sections at designated intervals, with a tissue chopper or by a hand razor (Weiler *et al.,* 1982).

When prepared and maintained properly, hippocampal slices or prisms retain electrical activity and neuronal integrity for several hours (Weiler *et al.* 1982). Intracellular recordings made from CA1 pyramidal cells in the hippocampal slice preparation have been shown to display those characteristics previously described for the intact preparation including EPSPs (excitatory post synaptic potentials), IPSPs (inhibitory post synaptic potentials), depolarising after potentials, frequency potentiation, double pulse facilitation and probable dendritic spikes (Scwartzkroin, 1975). Furthermore it has been shown that the quantitative measure of some passive membrane properties (for example cell resistance and rheobase level, important for establishing criteria by which to evaluate cell 'health' and electrode impalement) of CA1 pyramidal cells recorded from slices of guinea pig and rabbit hippocampus are in substantial agreement with measurements taken in intact preparations (Scwartzkroin, 1977). These studies thus demonstrate that cells in the

slices are not abnormal following the trauma suffered during its preparation and maintenance of the tissue *in vitro*. It would seem reasonable to conclude therefore, that the axon terminals of projection within the hippocampus retain their function and that hippocampal slices and prisms represent a feasible means of studying presynaptic mechanisms in this pathway.

Evidence from lesioning studies indicate that the cholinergic innervation of the hippocampus is almost entirely extrinsic. It was reported that septal lesions (made approximately 10-12 days prior to the experiments) destroyed cholinergic afferents to the hippocampus causing a significant reduction (>85%) of evoked ACh release (Mulder *et al.*, 1974) and according to Szerb's study completely abolished it (Szerb, 1977). Furthermore ChAT and AChE activity were also observed to be greatly reduced (75% and 60% respectively) (Mulder *et al.*, 1974). These results suggest that the release of ACh from intrinsic cholinergic neurons would account for only 15% at the most, of the total ACh release evoked in slices or prisms from the hippocampus. These results are supported by immunohistochemical staining for ChAT which revealed only a small incidence of cholinergic interneurones in the hippocampus (Sofroniew *et al.*, 1985).

Mechanisms of synthesis of ACh in brain slices or prisms

The biosynthetic enzyme choline acetyltransferase (ChAT), is not rate limiting and the rate of synthesis of ACh depends on the the availability of the precursors - choline and acetylcoenzyme A (acetylCoA). The source of acetylCoA for ACh synthesis is glucose or pyruvate. The supply of acetyl groups for ACh synthesis in the cytoplasm requires the translocation of acetylCoA from mitochondria to the cytoplasmic site where ChAT is available for ACh synthesis. The source of choline for ACh synthesis is partly extracellular since neurones do not synthesize choline *de novo* (Browning & Schulman, 1968) and also the synthesis of ACh is inhibited by

compounds such as hemicholinium-3 (HC-3) which inhibits high affinity choline transport.

Many studies have been concerned with the source and availability of choline and acetylCoA and their role in the regulation of ACh synthesis. There is evidence that synthesis and release of ACh *in vitro* can proceed without the addition of choline (Budai & Kasa, 1988, Weiler *et al.,* 1979; O'Regan & Collier, 1981). However there are also reports that the release of ACh is dependent on choline in the incubation medium, particularly during prolonged depolarization (Millington & Goldberg, 1982).

Although radioactive choline added to the incubating medium is incorporated into ACh in brain slices, a total dependence on extracellular choline for ACh synthesis has not been demonstrated. It has been suggested that in brain tissue *in vitro,* a net production of free choline occurs by release from its chemically bound form (lipid choline). Collier *et al.* (1972) showed, in brain slices, that bound choline can be utilized for ACh synthesis. ACh concentrations depleted by incubation in Na^+-free medium were restored without the addition of exogenous choline (Collier *et al.*, 1972). Furthermore it has been estimated from *in vivo* studies in the rat, that 12% of the brain choline originates from free choline in the plasma and 88% comes to the brain as lipid choline. It was concluded from this that the lipid choline which enters the brain pool from the plasma then contributes to the free choline available for ACh synthesis (Schuberth & Jenden, 1975). Nevertheless labelled choline in the medium successfully competes with unlabelled choline for ACh synthesis and it has been shown that 50-60% of the total ACh synthesized during a 60 min incubation is labelled (Molenaar *et al.,* 1973).

Subcellular fractionation of brain tissue has shown evidence of at least two compartments of ACh in the nerve endings; one contained in the vesicles and the other free in the cytoplasm and described as the cytoplasmic pool (Richter &

Marchbanks, 1971a). Isotopic labelling techniques have shown that slices or synatosomes when depolarized for prolonged periods of time, release newly synthesized ACh, from a rapidly turning over store in the tissue, which does not resemble the vesicular or cytoplasmic pools.

The subcellular localization of this store was investigated by measuring the ratios of labelled to total released ACh ('labelling ratio') in different subcellular fractions prepared from slices of brain (cortex) immediately after incubation with [^{3}H]choline. These ratios were compared with the values obtained for the ACh released during incubation in high KCl medium. Both in the synaptic vesicles and in the nerve ending cytoplasm, the labelling ratio of the ACh was much lower than that of the ACh released from the slices (Molenaar *et al.*, 1973). However the vesicle fraction has been reported to be heterogenous and it has been suggested that a small pool of vesicles highly labelled with [^{3}H]ACh is preferentially released (relative to the remaining larger vesicles found in the resting terminals) (Richter & Marchbanks, 1971a). The fact that such a pool of active vesicles has not yet been identified by subcellular fractionation methods described above, is thought to be due to either its lability, or the similarity of its physical properties to those vesicular or cytoplasmic fractions isolated (Molenaar *et al.*, 1973). It would seem unlikely that gross techniques like subcellular fractionation would reveal details at this resolution. However it is generally accepted that transmitter released by depolarization (electrical or high K^+) is of vesicular origin although it has been suggested that transmitters can be quantally released even from the cytoplasm in a Ca^{2+}-dependent manner (Tauc, 1982).

The relative contribution of newly synthesized ACh and longer-term (preformed) stores to evoked release has been investigated. Richter (1976) showed that during prolonged periods of depolarization (~90 min) the release of ACh was clearly biphasic. The peak rate of release gradually declined to a plateau level after about

10 min and was maintained for at least 60 min. It was observed that the peak release had a lower sensitivity to HC-3 (when added with the depolarizing K^+) and was also unaffected by added choline suggesting that the peak and plateau phases of release may be different. Richter put forward the idea that a larger portion of the peak than plateau phase might represent release of preformed ACh thus explaining its lower sensitivity to HC-3 during this period.

It was therefore decided to measure release of [^{3}H]ACh evoked by K^+ depolarization for 2 min. From the evidence above it was concluded that shorter periods of stimulation would be more likely to reflect preformed [^{3}H]ACh without a contribution from newly synthesized unlabelled ACh. K^+ depolarization was chosen as an alternative to electrical stimulation to exclude the effects of propagated action potentials in hippocampal interneurons and to localize the depolarization to nerve terminals. K^+ stimulated release of ACh has been shown to be insensitive to TTX (Benishin, 1990; Weiler *et al.,* 1984; Nordstrom & Bartfai, 1980) indicating that ACh release mediated by propagated action potentials does not occur during K^+ depolarization. The following experiments characterize the release system by examining the effect of varying concentrations of KCl as a depolarizing stimulus and also the effect of Ca^{2+} on evoked release of radiolabel.

METHODS

Preparation of tissue

Male Sprague Dawley rats (200-300g) were stunned and killed by cervical dislocation, followed by decapitation. The brain was removed and dissected on ice for the hippocampus. Hippocampal prisms were prepared by chopping the tissue at 0.2mm intervals in two planes at 45° angle, using a McIlwain tissue chopper. The prisms were added to ice cold superfusion medium (Krebs' solution) gassed with 5% CO_2/95% O_2 and thoroughly mixed with a vortex mixer in order to form a homogenous suspension. The tissue suspension was then centrifuged at 2400 rpm for 2 min and resuspended in superfusion medium. This procedure was repeated in order to eliminate any excess extracellular choline and finally resuspended in 3.5mls of gassed superfusion medium containing 20μl of [^{3}H]choline solution (giving a final concentration of 0.37μM; 1.268x10^7 DPM/ml) and incubated at 37°C for 40-45 min. Following centrifugation and resuspension as described above the prisms were then ready for superfusion.

Superfusion of the hippocampal prisms

The superfusion chambers were fitted with jackets through which water was circulated by a pump to maintain the chambers at 37°C. Each superfusion chamber contained a 7.5mm Swinnex Millipore filter (Millipore Corporation). An aliquot of 0.3ml of hippocampal prisms was then pipetted onto a disc of filter paper (Whatman 4) placed directly on the Swinnex Millipore filter bed. The hippocampal prisms were superfused with medium (Krebs' solution, continuously gassed with 95% O_2/5% CO_2, see Materials) at a rate of 0.25ml per min, using a Watson Marlow peristaltic pump. After an initial 60 min washout period, release experiments were initiated (t=0) and superfusate from each chamber was collected for successive 5 min intervals, in scintillation vials.

At the required times the superfusion medium was changed by transferring the input lines without stopping the pump, to superfusion medium containing the required concentration of KCl, drug or combination of drugs in Krebs' solution. [^{3}H]ACh release was initiated by superfusing the hippocampal prisms for two 2 min periods (S1 and S2 at t=9 and 34 min) with Krebs' solution containing high KCl concentrations. When a high KCl concentration (eg 30mM) was used, a corresponding reduction in NaCl concentration was made to maintain osmolarity.

The experiments with Ca^{2+}-free media were carried out in two different ways. Ca^{2+} was either excluded throughout the duration of the experiment (i.e after the initial washout period) or during the S1 and S2 stimulation only.

Determination of radiolabel in release experiments

The superfusion apparatus consisted of 12 chambers, accommodating 12 samples of hippocampal prisms (from a single rat), from which efflux of tritium was measured. The radioactivity in each 5 min sample was counted by adding 4ml of the water tolerant scintillation fluid 'Liquiscint' (see Materials) to each of the scintillation vials containing superfusate. After the second stimulation (S2), radioactivity remaining in the tissue was also determined. Each of the tissue samples was added separately to 4ml of NE260 scintillation fluid (which solubilizes tissue prisms) in scintillation vials.

All vials were left to stand overnight and then counted (5 min) for tritium in a Beckman LS 233 scintillation counter, wide window for tritium, counting efficiency 35%. In preliminary experiments it was shown that the counting efficiency in the two scintillation fluids is the same and that there were no variations in quenching caused by volumes of superfusion medium ranging from 0.5-1.5ml.

Release in each 5 min superfusate was expressed graphically as CPM eg. Figure 3.1a. It was also normalized as fractional release (%, eg. Figure 3.1b), calculated according to the following formula.

$$\frac{\text{CPM released per fraction}}{\text{Total CPM released + CPM remaining on filter after superfusion}} \text{X100\%}$$

After the 2 min K^+ stimulation (S1 or S2), radioactivity increased in the 3 subsequent 5 min superfusates eg. Figure 2.1b. The corresponding fractional releases for each tissue sample separately were corrrected for baseline fractional release and then summed to give total fractional release for that particular stimulation (S1 or S2). This was done by subtracting the value of the fractional release calculated for the 5 min superfusate immediately prior to the K^+ stimulus. The corrected total fractional releases in the S2 and S1 stimulations were then calculated for each sample separately.

Thus the result of any typical experiment is four separate and invariate control S2/S1 ratios and four separate and invariate ratios for drug superfused tissue. From these control and drug-treated groups, the mean and standard error of the mean (SEM) ratio was then calculated and displayed graphically. Thus the SEMs of the S2/S1 ratios displayed throughout this thesis represent variations between tissue samples and also between rats where experiments were repeated on different occasions (usually N=4, see statistical analysis), eg. Figure 3.3.

It should be noted that where fractional release plots are shown (Figures 2.1b, 2.2b, 2.3b, 3.1b, 3.2b, 3.4b, 3.6b, 3.9b & 4.13b), fractional releases at each time point for the replicate tissue samples involved (from 1 rat, i.e 1 experiment), are represented for convenience as mean ± SEM, even though the individual values were kept

segregated for the calculation of individual S2/S1 ratios. The fractional release plots therefore cannot be used to calculate the final S2/S1 ratios and their SEMs, and are displayed simply to show the "signal to noise" ratio in typical experiments. The SEMs for the fractional release plots indicate the variations between quadruplicate aliquots of a single tissue sample (from 1 rat, i.e 1 experiment) and largely reflect variations in the sampling of suspensions of prisms in superfusion medium. These suspensions sediment rapidly after mixing with a vortex mixer so that good aliquot reproducibilty is difficult to achieve. Nonetheless individual S2/S1 ratios calculated as described above, will be independent of between-aliquot variations since S1 and S2 in each ratio refer to the same aliquot.

Experimental design

The 12 superfusion chambers were divided into 3 groups for comparison - i.e a control group and 2 test groups, each comprising 4 superfusion chambers, thus giving 4 replicate S2/S1 ratios for each group. Each experiment was repeated 4 times (N=4) unless otherwise stated.

Statistical analysis

Analysis of variance (ANOVA)

Data (S2/S1 ratios) from control and the two drug-treated groups from experiments done on different occasions (N=4 rats, i.e 4 experiments) were analyzed as a two-way analysis of variance (MINITAB, statistical package, IBM pc). In situations where significant differences in variances were noted between control and drug-treated groups ($P<0.05$) probability levels were assigned using a subsidiary t-test (MINITAB). Throughout, there was no interaction between drug treatments and experiments (rats).

The mean S2/S1 ratios and their SEMs, eg. Table 3.1 and Figure 3.3 were calculated in one of two ways depending on the results of the two-way analysis of

variance. Where there was no difference in variance between experiments (rats) i.e $P>0.05$, the overall mean ± SEM was calculated from the total number of replicates (n=16 or less) pooled from all 4 experiments. Where $P<0.05$, the overall mean ± SEM was calculated from the individual means for each separate experiment (N=4).

It was only possible to test 2 drug (test) groups and a control in any one experiment (i.e 3x4 superfusion chambers). Therefore in order to present all the data on a single concentration-effect graph (eg. Figure 3.3), the mean S2/S1 values from each of the separate control groups were used to calculate the overall mean control value and the associated standard error (N>4).

t-tests

It is known that t-tests when used for small samples require that the two population standard deviations be approximately equal. Therefore in situations where the sample standard deviations differ appreciably (i.e when one is twice the other or more) an alternative method using adjusted means and standard errors must be used to calculate the t value.

Adjusted means provide a mean that is unaffected by the differing number of observations in the different experiments and also adjusts for the possibility of interactions. Thus the adjusted mean is the main response to that treatment, independent of any possible experimental differences either directly or in interaction and is essentially a mean of means rather than a grand mean. Therefore the means compared are not simple unweighted means of the observations. Instead, the mean of each treatment in each experiment was found and the average of those means for a particular treatment was taken.

However in order to estimate the standard error (SEM) an estimation of the population variance is required. The more data available, the more sensitive the t-

test will be - i.e better able to detect differences. In the standard two sample t-test, the data from both samples are used to estimate the variance (σ^2). In this case however, even though the comparison is between two groups, the data from all 3 groups are used to estimate σ^2. In fact the estimate of σ^2 used, is the error mean square from the ANOVA table calculated by Minitab. This value σ^2, is used by Minitab to give the standard deviation (SD) = standard error of each mean.

The following equation was used to calculate t values, which were then compared with calculated values tabulated in t-distribution tables for significance. The error degrees of freedom as shown from the analysis of variance of unweighted means (MINITAB) was used to assign probability levels - $P>0.05$ or $P<0.05$, 0.01, 0.001 or 0.0001.

$$t = \frac{meanA - meanB}{\sqrt{(SDA)^2 + (SDB)^2}}$$

MATERIALS

Animals

Male rats of the Sprague-Dawley species weighing 200-300g were used for all the experiments described throughout the thesis. These were obtained from the colonies bred and maintained by the Biological Services Unit at King's College London, Kensington Campus.

Radiochemicals

From Amersham International plc, Buckinghamshire, England.

[Methyl-^{3}H]choline chloride in ethanol solution; specific activity 555GBq/mmol (15Ci/mmol).

Solutions

Superfusion medium (Krebs' solution)

NaCl 121mM; KCl 1.85mM; KH_2PO_4 1.17mM; $MgSO_4$ 1.17mM; glucose 11.1mM; $NaHCO_3$ 25mM; $CaCl_2$ 1.22mM, pre-equilibrated with 95% O_2/5% CO_2 gas mixture.

High K^+ Krebs' solution

NaCl 98mM; KCl 30mM; KH_2PO_4 1.17mM; $MgSO_4$ 1.17mM; glucose 11.1mM; $NaHCO_3$ 25mM; $CaCl_2$ 1.22mM, pre-equilibrated with 95% O_2/5% CO_2 gas mixture.

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Scintillants

From National Diagnostics, Hessle, Hull.

'Liquiscint'.

From NE technology, Sighthill, Edinburgh.

General purpose scintillator for internal counting of aqueous and non aqueous samples- NE 260.

RESULTS

Preliminary studies showed no difference in the counts for tritium observed between radiolabel (a fixed volume) counted in 4ml of Liquiscint or 4ml of NE260. Furthermore, varying the volume of Krebs' solution (0.5, 1.0 and 1.5ml) containing fixed amounts of radiolabel also did not affect the counts, indicating that quenching does not vary in the volume of superfusate collected in 5 min at a flow rate of 0.25ml per min.

1. The effect of varying the depolarizing concentration of K^+

The collection of 5 min fractions began after a washout period of 60 min with superfusion medium (Krebs' solution), following preliminary experiments which showed that approximately 60 min of pre-superfusion was neccesary to wash out the excess radiolabel and to reduce the resting release of radioactivity to a steady level.

When the prisms were superfused (stimulated) with high K^+ Krebs' solution containing normal Ca^{2+} concentrations (1.22mM) for 2 min, a marked increase in the release of radioactivity relative to spontaneous/basal levels was observed (Figure 2.1). The appearance of increased radioactivity began approximately 5 min after K^+ stimulation. Counts of succesive 5 min samples showed that radiolabel collected in the superfusion medium reached maximum after 5 to 10 min.

As Figure 2.1 shows the amount of radioactivity released was dependent on the concentration of K^+ used. The release of radioactivity was appreciably greater with 50mM KCl during the S1 stimulation than with 10 or 30mM. However the peak release observed during the S2 stimulation (with 50mM) was comparable to that of the S2 stimulation observed with 30mM K^+. The mean ± SEM S2/S1 ratios corresponding to the data shown in Figure 2.1 are 10mM K^+ S2/S1 0.910 ± 0.213, 30mM K^+ S2/S1 0.750 ± 0.032 and 50mM K^+ S2/S1 0.657 ± 0.048. It is noted that

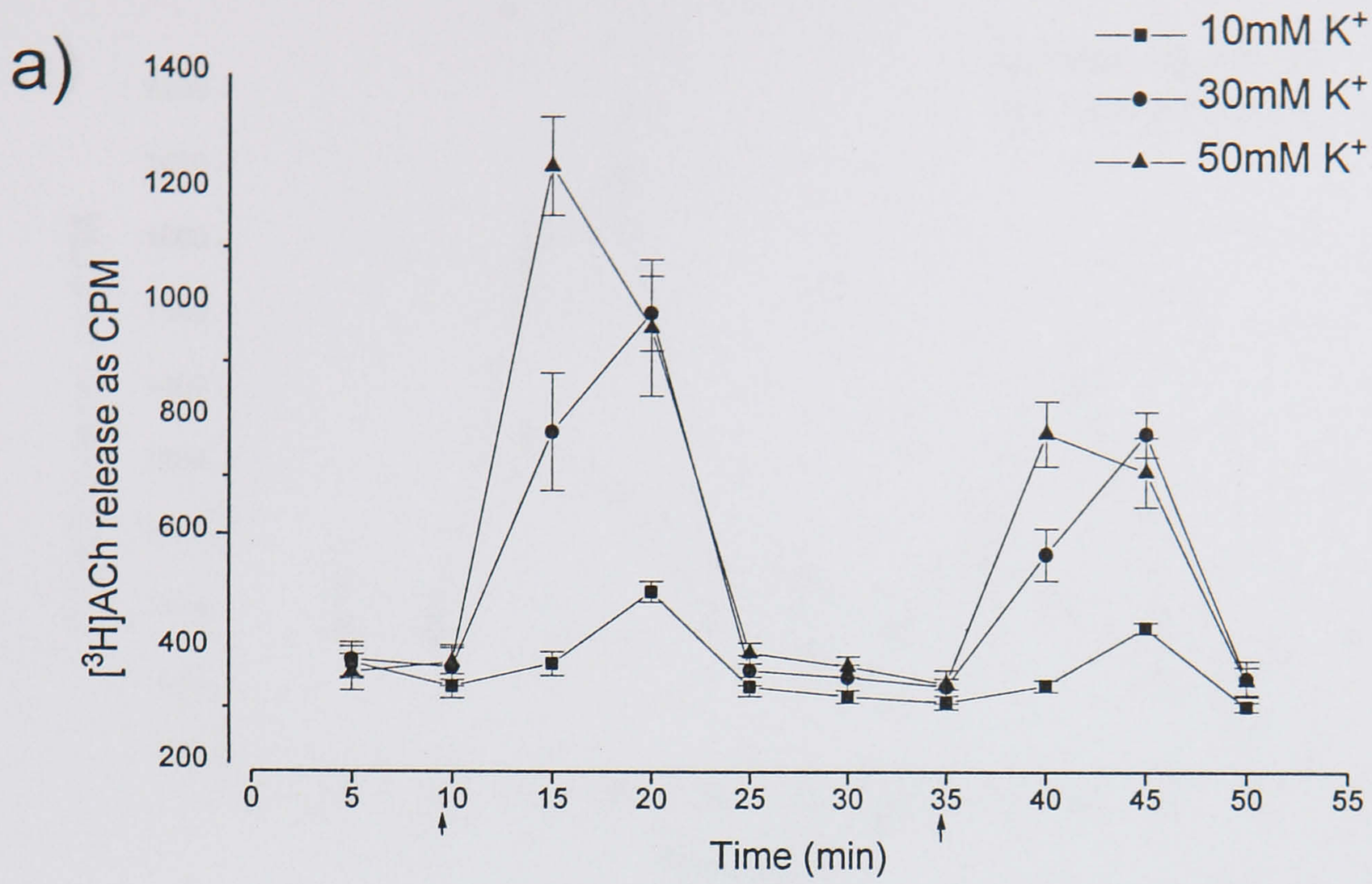
the trend is for the S2/S1 ratio to decline with increasing K^+ concentration although this did not prove statistically significant.

2. The effect of varying the stimulation time

The effect of varying stimulation times (2 min versus 3 min) is shown in Figure 2.2 where 30mM K^+ was used. The mean ± SEM of the S2/S1 ratios corresponding to the data shown in this Figure are, 2 min stimulation S2/S1 0.731 ± 0.069 and 3 min stimulation S2/S1 0.579 ± 0.048. The mean S2/S1 ratio observed when K^+ stimulation was 2 min (S2/S1 0.727 ± 0.018, N=11) was significantly greater ($P<0.001$) than that for 3 min stimulations (S2/S1 0.587 ± 0.016, N=5).

3. The effect of Ca^{2+} on release of radiolabel

Superfusion with Ca^{2+}-free medium began after the initial washout period. This caused a gradual decrease in spontaneous/basal release - Figure 2.3. Under these conditions, stimulation with Ca^{2+}-free high K^+ solution showed no release of radioactivity during S1 or S2 stimulations. However as Figure 2.3 shows, omission of Ca^{2+} only in the high K^+ superfusing medium caused a marked decrease in radioactivity during S1 and S2 stimulations.



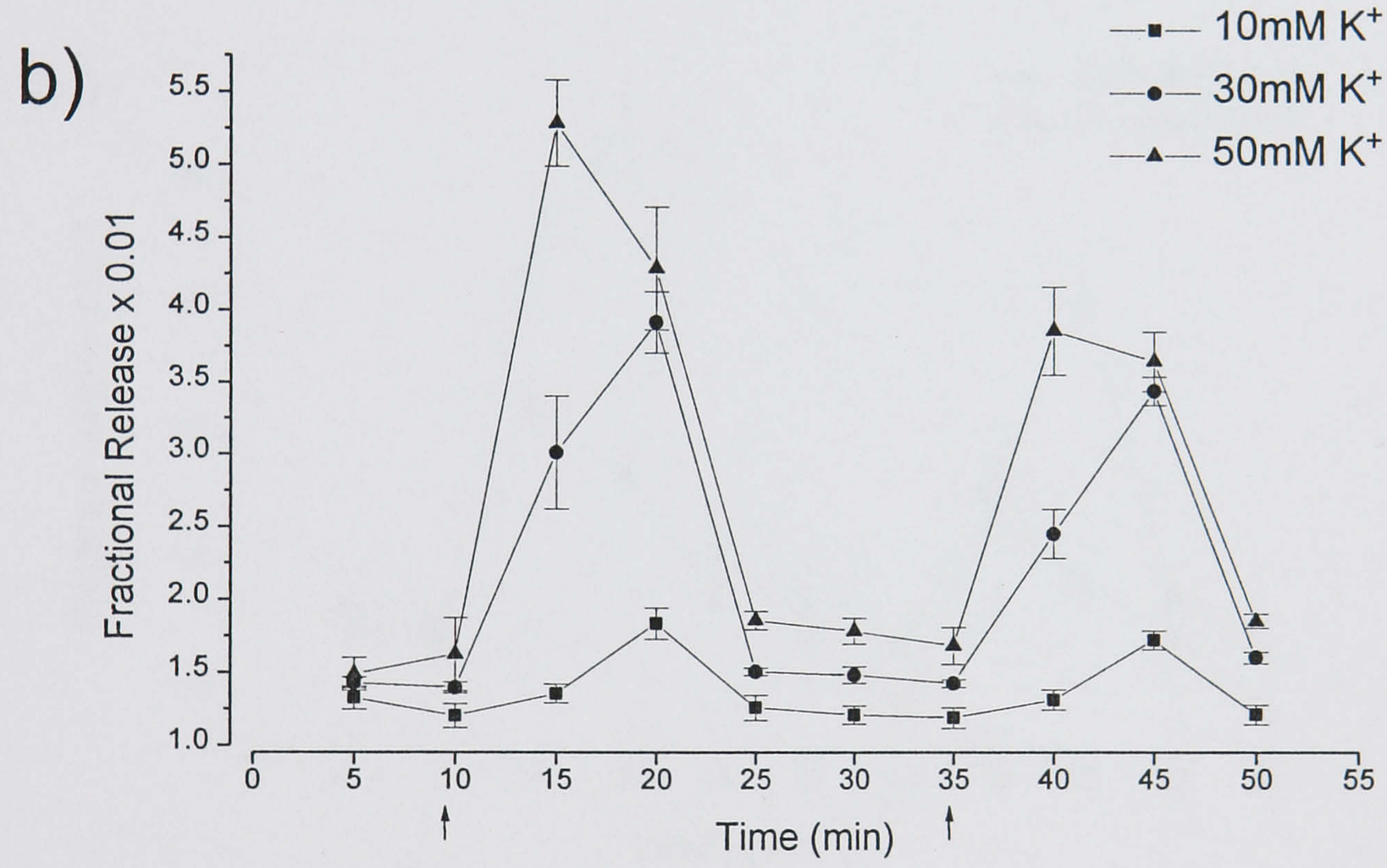


Figure 2.1 The release of radiolabel from rat hippocampal prisms evoked by high K^+ concentrations. The prisms were preloaded with [^{3}H]choline (see Methods for details) and stimulated twice (S1 and S2) for 2 min with different concentrations of K^+ (10, 30, 50mM). The point of application of K^+ is indicated by arrows on the X-axis. Each point represents the mean ± SEM of replicate samples obtained from a typical experiment expressed as:

a) CPM

b) fractional release

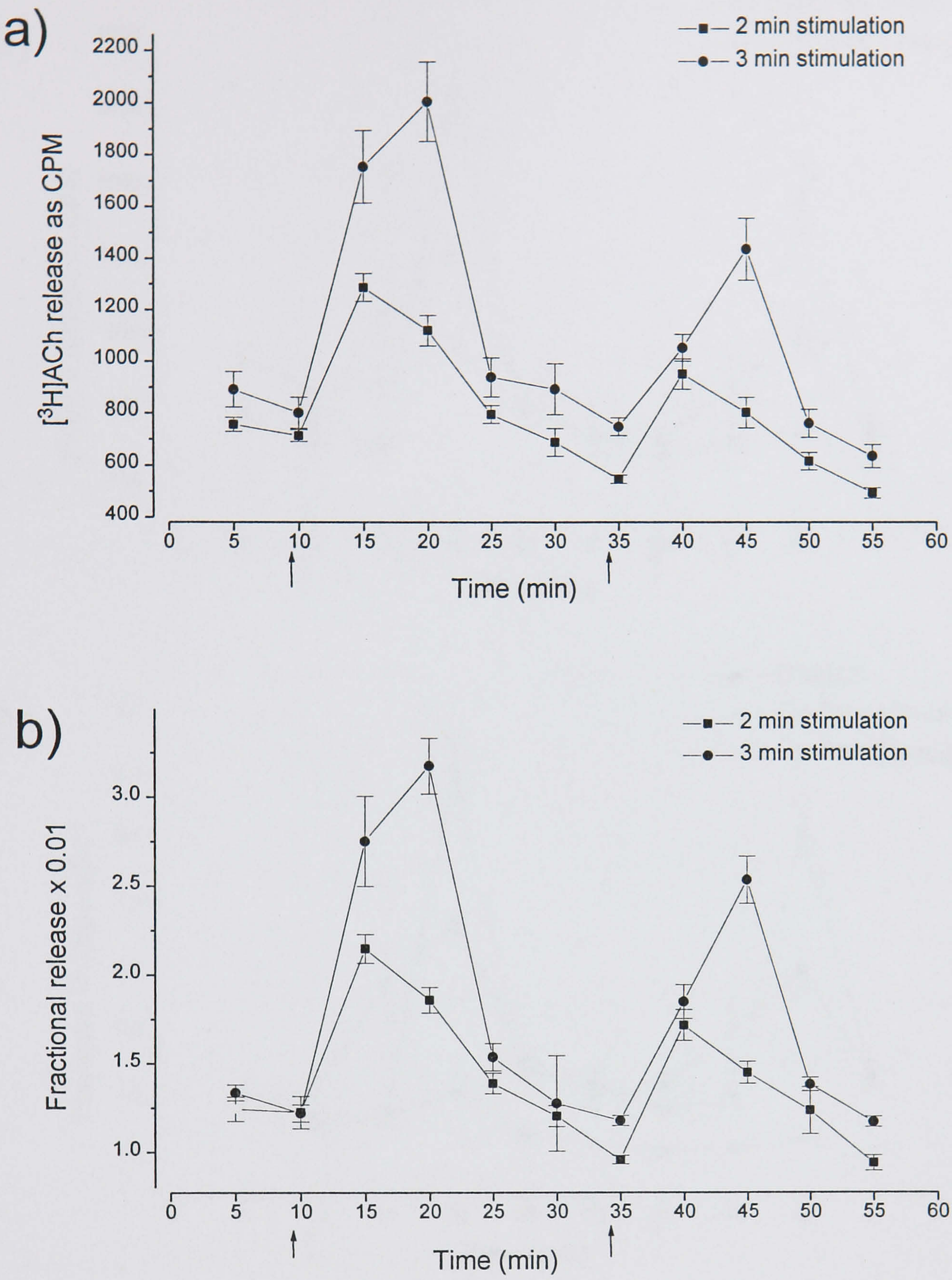


Figure 2.2 The release of radiolabel from rat hippocampal prisms evoked by high K^+ concentrations - the effect of varying stimulation times.

The prisms were preloaded with [^{3}H]choline (see Methods for details) and stimulated twice (S1 and S2) for 2 min or 3 min with 30mM K^+. The point of application of K^+ is indicated by arrows on the X-axis. Each point represents the mean ± SEM of replicate samples obtained from a typical experiment expressed as:

a) CPM

b) fractional release

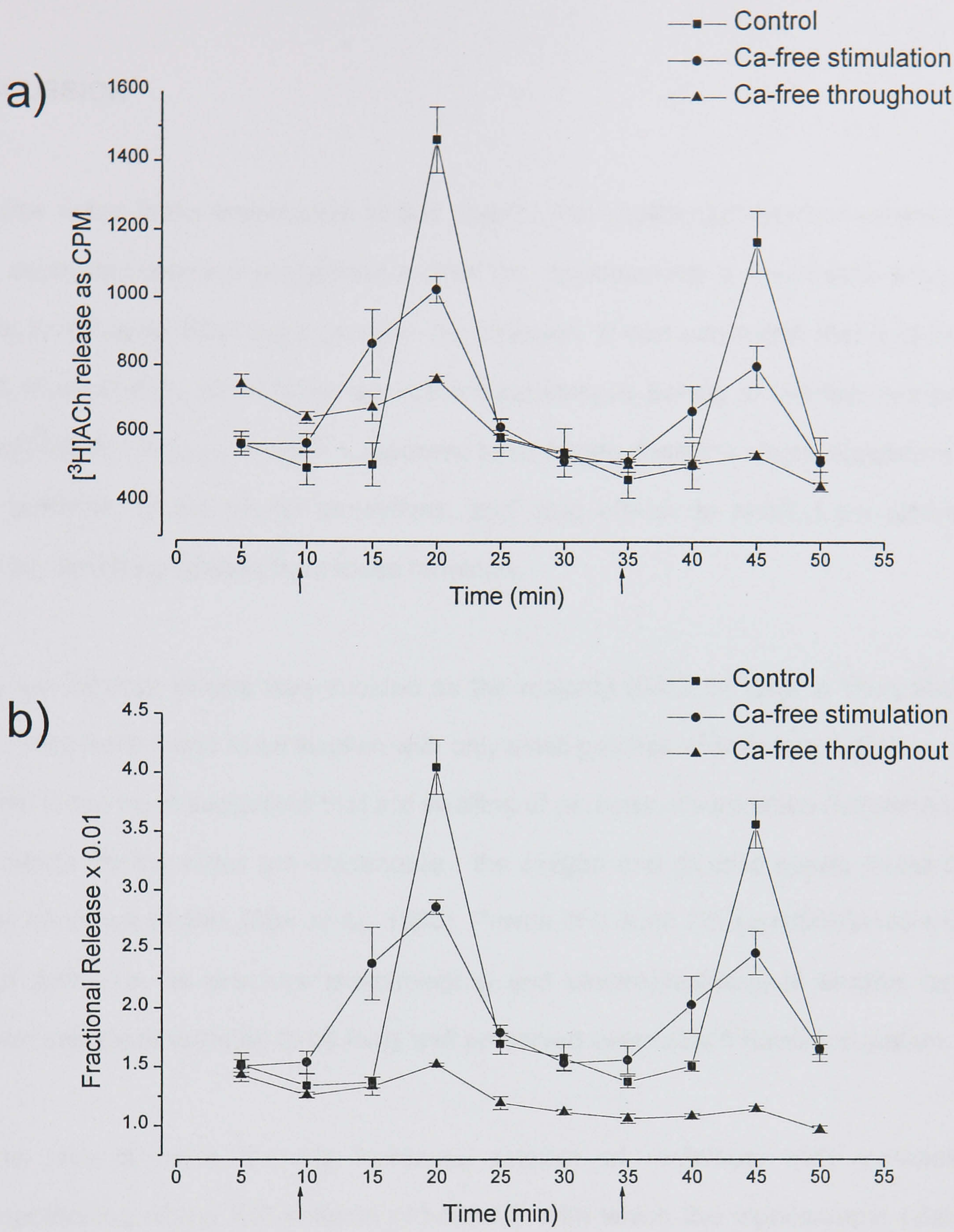


Figure 2.3 The release of radiolabel from rat hippocampal prisms evoked by high K^+ concentrations - the effect of Ca^{2+}.

The prisms were preloaded with [^{3}H]choline (see Methods for details) and stimulated twice (S1 and S2) for 2 min with 30mM K^+. The point of application of K^+ is indicated by arrows on the X-axis. Ca^{2+} was omitted either throughout the experiment (i.e in the superfusion medium and high K^+ Krebs' solution), OR only in high K^+ Krebs' solution (i.e in S1 and S2 only). Each point represents the mean ± SEM of replicate samples obtained from a typical experiment expressed as:

a) CPM

b) fractional release

DISCUSSION

As was noted in the Introduction to this chapter, the cholinergic neuronal content of the septo-hippocampal projections makes the hippocampus a favourable area of brain for studying cholinergic synaptic transmission. It was concluded that less than 15% of cholinergic nerve terminals in the hippocampus belong to intrinsic neurons. Thus [^{3}H]ACh release evoked is assumed to be largely from the single population of the terminals of the septal projections, and drug effects to result from discrete actions modifying release from these terminals.

The use of thick prisms was avoided as the majority (98%) of cells in thick slices (~0.7mm) were found to be swollen with only small patches of cells intact (Bak *et al.,* 1980). It has been suggested that the swelling of neurons occurs when maintenance conditions for the slices are inadequate - the oxygen and glucose supply being the most important factors (Bak *et al.,* 1980). Prisms of 0.2mm (200μm) thickness were used therefore, as previous morphological and electrophysiological studies have shown thin slices (striatal) to be fairly well preserved even after 5 hours incubation.

There was a trend towards increased release of radiolabel with increasing concentrations of the KCl solution (10-50mM), with which the hippocampal prisms were superfused (Figure 2.1). Changes in ionic strength are not responsible for this effect as it has been shown that 33mM Tris-HCl did not have any effect on [^{3}H]ACh release when compared with an identical concentration of K^+. This concentration of K^+ also does not affect the activity of lactate dehydrogenase released into the superfusate, which is an indicator of the integrity of the tissue (Richter & Marchbanks 1971b). Changes in K^+ concentration are however responsible for depolarization of nerve terminals in the prisms and cause neurotransmitter release. According to the Nernst equation, a K^+ concentration of 30mM would be expected to cause a 55-60mV depolarization. This would be sufficient to activate voltage-operated calcium

channels and to initiate [^{3}H]ACh release from cholinergic nerve terminals in the slices. The relationship between nerve terminal depolarization, Ca^{2+} channel activation and ACh release is considered further in Chapter 5

It is notable that while the S1 release evoked by 50mM K^+ is appreciably greater than that evoked by 30mM K^+ the S2 releases are comparable. An interpretation of this observation is that depletion or dilution of releasable pool is greater after the stronger S1 stimulus, thus reducing the availability of radioactivity for release in the S2 stimulus. If this is so drug-induced potentiation of release (Chapters 3-6) could well be underestimated, particularly at 50mM concentrations of K^+. Furthermore drug-induced inhibition of release would be difficult to observe with 10mM K^+ (Figure 2.1). It seemed therefore, that 30mM K^+ was the most favourable concentration for observing inhibition and facilitation of [^{3}H]ACh release.

It can be argued as above, that the decline in S2/S1 ratio with increasing K^+ (Figure 2.1) is due to increased release during the S1 stimulation, which causes a greater depletion of the releasable pool of [^{3}H]ACh. Thus there remains progressively less to drive release in the S2 stimulation. Furthermore an increasing S1 release would lead to an increase in uptake of unlabelled choline. This unlabelled choline would be acetylated and dilute the pool of releasable [^{3}H]ACh. Thus dilution and depletion together may account for the fractional S2/S1 ratio (0.6-0.85) observed with the radiolabelling technique used throughout the work described in this thesis.

From the above argument it would appear that the measurement of unlabelled ACh release would reflect only depletion of the releasable pool of ACh. Indeed it was observed by Richter (1976) that K^+ (50mM) evoked release (from prisms) of unlabelled ACh during the S2 stimulus was ~ 70% of S1. However Beani *et al.*, (1984) reported an S2/S1 ratio of 1 for electrically evoked release of unlabelled ACh. As mentioned in the Introduction to this chapter, high K^+ has been shown to cause a

greater, more prolonged release of ACh than electrical stimulation (Hadhazy & Szerb, 1977). This may be likely to cause a greater depletion, thus explaining the discrepancy between these two studies.

Previous accounts of the superfusion technique specified flow rates of 0.25 to 0.6 ml per min (Murrin *et al.,* 1977; Raiteri, 1974; Hadhazy & Szerb, 1977; Marchi *et al.,* 1981; Mulder *et al.,* 1974). From preliminary experiments (data not shown) it was concluded that potentiation of release by tacrine (30μM) (Chapter 3) was not significantly changed by flow rates of 0.25 or 0.4ml per min or by K^+ concentrations in the range 20 - 30mM. However it was found that 3 min stimulation of the prisms with 30mM K^+ gave a significantly smaller control S2/S1 ratio than did 2 min stimulations (see Results and Figure 2.2). Thus a stimulation time of 2 min exposure to 30mM K^+, at a superfusion rate of 0.25ml per min was employed throughout the course of the work described in this thesis, where evoked release is described.

It is well known that Ca^{2+} is essential for the release of neurotransmitter from nerve terminals and as Figure 2.3 shows, its omission from the superfusion and high K^+ medium abolished the K^+-evoked release of radiolabel. These results confirm the Ca^{2+}-dependence of [^{3}H]ACh release in these experiments. Excluding Ca^{2+} from only the high K^+ medium did not have such a dramatic effect. This is possibly due to residual Ca^{2+} within the nerve terminals and tissue interstices since it is unlikely that a Ca^{2+}-free environment could have been attained in a period of 2 min. The use of Ca^{2+} chelators such as EGTA however was avoided in this study.

Electrophoretic separation of [^{3}H]choline from [^{3}H]ACh was not possible at the low levels of release at which the experiments were carried out (approximately 1000cpm, 0.1pmol). There is however substantial evidence that the increase in efflux of radiolabel evoked by electrical or K^+ stimulation is in fact due to [^{3}H]ACh (Pohorecki *et al.,* 1988; Mulder *et al.,* 1974; Beani *et al.,* 1984; Richardson & Szerb, 1974;

Szerb, 1977). Moreover [^{3}H]choline release has been shown to be unaffected by K^+ (Richter, 1976; Weiler, 1989; Wonnacott & Marchbanks, 1976) which suggests that the increased radioactivity observed must be the result of [^{3}H]ACh release.

CHAPTER 3

Potentiation of [^{3}H]ACh release by ChE-inhibitors and HC-3

INTRODUCTION

The discovery of reduced concentrations of markers for cholinergic neurones, in the cortex and hippocampus from patients with Alzheimer's disease (Davies & Maloney, 1976) led to the use of cholinomimetic agents as a therapy for these patients. Oral loading of precursors such as choline (Etienne *et al.*, 1978) and phosphatidylcholine (lecithin)(Etienne *et al.*, 1981) however, did not have appreciable effects. Agonist therapy too, proved to be unsatisfactory (Spiegal, 1989). Enhancement of the action of the naturally released agonist from the surviving cholinergic neurones by a cholinesterase inhibitor appears to be the most favourable strategy so far, in the treatment of Alzheimer's disease, although it would not discriminate between the variety of cholinoceptor subtypes now known to exist in the CNS.

Eserine, a carbamate inhibitor of cholinesterases (ChE), was the first of its kind to be used in the treatment of patients with Alzheimer's disease. Given orally, its effect was found to be too brief and irregular. It is not therefore considered an effective therapeutic agent because of its unfavourable pharmacokinetic profile and the insufficient inhibition of ChE allowed by its narrow therapeutic window (Thal *et al.*, 1988). More recently, Summers *et al.*, (1986) re-introduced tacrine (1,2,3,4-tetrahydro-9-aminoacridine or THA), a potent and reversible inhibitor of butyryl- and acetyl-ChEs, as an agent likely to be of therapeutic benefit to some patients with Alzheimer's disease. Tacrine has been shown to be a longer acting ChE-inhibitor with a wider therapeutic window than eserine. Despite numerous criticisms about Summer's trial, tacrine recently won unanimous approval from the FDA following the data presented by Farlow *et al.*, (1992) which showed that it was superior to placebo in alleviating the symptoms of Alzheimers disease. It was therefore thought worthwhile by many clinicians, to investigate tacrine more rigorously.

Tacrine has featured in a number of studies using brain slices or synaptosomes. There have been some reports that it inhibits the release of [^{3}H]ACh from electrically stimulated slices of rat striatum, cortex and hippocampus (Drukarch *et al.,* 1987; Loiacono & Mitchelson, 1990; Potter *et al.,* 1989) and also that it increases release (Loiacono & Mitchelson, 1990; Potter *et al.,* 1989), although this effect was not always supported by the statistical data. It was speculated that inhibition involved muscarinic receptors and increase of release involved nicotinic receptors, both types being located presynaptically. More recently Umeda & Sumi (1990), using a fixed volume (non-superfusion) technique, have provided clearer evidence that tacrine as well as eserine (Ishi & Sumi, 1992) increases basal and K^+-evoked release of unlabelled ACh from hippocampal and striatal slices.

The effects of tacrine and eserine have also been studied using human brain slices obtained at post mortem (Nilsson *et al.,* 1987; Nordberg *et al.,* 1989) with delays of less than 22 h. In non-Alzheimer cortical tissue (control), tacrine (100μM) or eserine (100μM) significantly decreased the K^+-stimulated release of [^{3}H]ACh. In Alzheimer samples, the evoked [^{3}H]ACh release was intrinsically less than in the control tissue, but it was enhanced to control values in the presence of tacrine or eserine at 100μM. The nicotinic antagonists dihydro-β–erythroidine (DHβE) or mecamylamine, were shown to counteract the enhancement of release caused by tacrine in the Alzheimer samples whereas in non-Alzheimer samples, these antagonists were unable to change the tacrine effect (Nordberg *et al.,* 1989). Atropine, which increased the evoked release of [^{3}H]ACh from the Alzheimer samples, was also shown to abolish the effect of tacrine in the same tissue. This study indicates a complex mechanism of action for tacrine, involving both muscarinic and nicotinic receptors and this is indeed supported by receptor competition studies, where tacrine is shown to displace [^{3}H]nicotine and [^{3}H]QNB binding (Nilsson *et al.,* 1987).

Investigations of the action of tacrine on peripheral ACh release, however, seem to be more supportive of its role as a cholinomimetic, compared to the observations made with CNS tissue. Tacrine has been shown to increase the spontaneous quantal release of ACh at the neuromuscular junction (Thesleff *et al.,* 1990), as well as the quantal release evoked by K^+ depolarisation (Provan & Miyamato, 1990) and by nerve stimulation (Harvey & Rowan, 1990). Tacrine was also shown to be effective in the chicken heart (Lindmar & Loffelholz, 1990), where it enhanced the ACh release caused by field stimulation. As discussed above, evidence of its effectiveness as a cholinomimetic in the CNS has not been widely reported, especially in the absence of other drugs.

In addition to the inhibition of AChE, tacrine has also been shown to have multiple actions in the CNS. In rat brain, it has been reported to increase the basal release of [^{3}H]NA (noradrenaline) and [^{3}H]5HT (5-hydroxytryptamine) from cortical slices and [^{3}H]DA (dopamine) from striatal slices (Drukarch *et al.,* 1988; Robinson *et al.,* 1989; Baldwin *et al.,* 1991) and also to inhibit both forms of monoamine oxidase (MAO) (Nordberg *et al.,* 1989; Adem, 1992). It has also been shown to inhibit the K^+-evoked release of [^{14}C]GABA (γ-aminobutyric acid), from cortical slices (De Belleroche & Gardiner, 1988). Tacrine displays affinity for muscarinic and nicotinic cholinoceptors (Drukarch *et al.,* 1988; Nilsson et al., 1987; Nordberg *et al.,* 1989) and shows about a 10 fold greater affinity for muscarinic cholinoceptors compared to nicotinic cholinoceptors (De Sarno *et al.*, 1989; Nilsson *et al.,* 1987). It also interacts with other receptors such as those for phencyclidine (Albin *et al.,* 1988) and at high concentrations, adenosine receptors (Freeman *et al.,* 1988). Furthermore, tacrine has a structural similarity to 4-aminopyridine, and similar to this compound, blocks K^+ channels in a variety of tissues (Osterrieder, 1987; Halliwell & Grove, 1989; Rogawski, 1987; Griffith & Sim, 1990; Drukarch *et al.,* 1987; Chaki *et al.*, 1991). It also blocks Na^+ and Ca^{2+} currents in rat dorsal root (Kelly *et al.,* 1991) and superior cervical sympathetic ganglia (Marsh *et al.,* 1990).

Following renewed claims supporting the clinical usefulness of tacrine in Alzheimer's disease, the aim of the study described in this chapter was to clarify the effect of the drug, on K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms and to compare its effects with three other classical, ChE inhibitors - eserine, neostigmine and edrophonium. Eserine, a carbamate ester was chosen because of its previous trials in the treatment of Alzheimer's disease. It has also been used in the treatment of myasthenia gravis and glaucoma and also experimentally in the measurement of ACh release *in vitro*, where ChE inhibitors are frequently added to protect the ACh released, from hydrolysis. Eserine is predominantly positively charged at physiological pH (approximately 76%) and combines with the anionic site of the ChE in a similar way to neostigmine.

Neostigmine, also a carbamate ester is completely ionized in aqueous solution, because it is a quaternary ammonium compound. Since the blood-brain barrier is largely impermeable to quaternary ammonium compounds, neostigmine and related compounds are without significant effect on the CNS. These carbamyl esters resemble ACh and react with the esteratic site of ChE in a similar way, except that hydrolysis occurs at a much slower rate. Edrophonium however, a quaternary ammonium compound lacks the carbamyl ester group and cannot be hydrolyzed by ChE. Instead it slows down the rate of hydrolysis of ACh by combining very rapidly at the anionic site of the ChE. It has a very rapid dissociation rate on dilution which limits its use clinically. Like edrophonium, tacrine also lacks a carbamyl group and binds to the anionic site of the ChE. Neostigmine and edrophonium were chosen in the present study because of their selective extracellular actions, due to the positively charged nitrogen atom in these molecules. It was anticipated that tacrine and eserine might have additional intracellular actions.

The hippocampus was chosen for study because it is known to receive cholinergic innervation and is one of the major areas affected in Alzheimer's disease (see

Chapter 1). Although a few cholinergic neurones may be intrinsic to the hippocampus, greater than 85% of cholinergic hippocampal fibers arise from areas outside the hippocampus which include the medial septum and the basal forebrain nuclei. It was therefore considered reasonable to assume that drug effects, on ACh release from hippocampal prism preparations, would reflect predominantly actions at the level of the nerve terminal, rather than actions requiring propagated nerve action potentials.

Furthermore DNA cloning techniques have revealed the existence of five different genes coding for the muscarinic receptors m1, m2, m3, m4 and m5 which are expressed in the brain. Studies with complementary nucleic acid sequences which are able to hybridize with parts of muscarinic receptor mRNA, either with Northern blots of mRNA from tissues, or *in situ* hybridization, along with immunoreactivity studies have broadened existing knowledge regarding the anatomical localization of muscarinic receptors. The hippocampus has been shown to contain mRNA for all five (m1-m5) muscarinic receptors and also shows immunoreactivity for the corresponding antireceptor antibodies (Brann *et al.,* 1993; Ladinsky, 1993; Vilaro *et al.,* 1993).

Immunohistochemical studies have also localized brain nicotinic cholinoceptors using specific antibodies. It was observed that the distribution for CNS nicotinic cholinoceptors was very similar to that of radiolabelled nicotinic agonist binding sites (Lukas & Bencherif 1992). There is evidence for the existence of multiple types of nicotinic cholinoceptors in the CNS on the basis of the identification of a variety of subunit-encoding genes and messenger RNAs in brain tissue and also on the basis of long recognized complexities in the pharmacological/physiological characteristics of receptors mediating nicotinic cholinergic responses in the brain. Seven α-subunit genes (α2 to α**9**) and four β-subunit genes (β2-β**4**) have been identified. While α7 (and rodent α4) subunits are the only subunits that are apparently capable of forming functional homo-oligomeric channels, any pairwise combination of α2,α3 or α4 with

β2 or β4 have been shown to generate ion channel activity when expressed in the xenopus oocyte system (Lukas & Bencherif 1992; Role, 1992). From the foregoing, it is clear that the hippocampus is equipped to display a full range of cholinergic mechanisms and is also anatomically relevent to Alzheimer's disease.

The presence of monoaminergic innervation of the hippocampus has also been established (Lindvall & Bjorklund, 1974) and evidence exists that manipulations of the dopaminergic, noradrenergic and serotonergic systems innervating the forebrain have pronounced effects on *in vivo* ACh release in the hippocampal formation (Robinson & Vanderwolf, 1978; Nilsson *et al.,* 1992). Hippocampal ACh is known to be tightly linked to the behavioural state of the animal and these observations indicate that the monoaminergic afferents to the septum and the hippocampal formation may play an important role in the mediation of these brain stem regulatory influences.

As tacrine is also known to affect the release of monoamines in the CNS (Drukarch et al., 1988; Robinson *et al.,* 1989), a part of the study described in this chapter was also to investigate the extent to which monoamines are implicated in the effects of tacrine on [^{3}H]ACh release.

METHODS

1. Release experiments

Preparation of tissue

Hippocampal prisms were prepared and preloaded with [^{3}H]choline as described in Chapter 2 - General Methods and Materials. When preloading the prisms with the radioactive monoamines [^{3}H]NA, [^{3}H]DA or [^{3}H]5-HT, a monoamine oxidase inhibitor (MAO-inhibitor) was added in order to prevent the breakdown of these neurotransmitters. The prisms were incubated with 5μM pargyline 5 min prior to to the addition of the radiolabelled [^{3}H]NA or [^{3}H]5-HT and then incubated for a further 30 min at 37°C. The final concentration of [^{3}H]NA and [^{3}H]5-HT in the hippocampal prism preparation was 0.21μM ($1.86x10^7$ DPM/ml) and 0.61μM ($2.46x10^7$ DPM/ml). When [^{3}H]DA was used at a final concentration of 0.07μM ($6.5x10^6$ DPM/ml), 10μM nialamide was also included in the incubation medium, which was otherwise the same as the superfusion medium (see Chapter 2).

Reserpinization of the prisms

In some experiments the prisms were also treated with reserpine to deplete monoamine stores. Reserpine (300nM) was added with the radiolabelled amine (or [^{3}H]choline) and the procedure continued as described above. Superfusion of the prisms was carried out as described in Chapter 2.

2. [^{3}H]QNB binding experiments

Preparation of tissue

Whole membrane preparations were used in radioligand binding experiments. Male Sprague-Dawley rats (250-300g) were stunned, cervically dislocated and decapitated. Their brains were rapidly removed and dissected on ice for the

hippocampus. The hippocampi thus obtained were added to 39 volumes (w/v) of ice-cold 0.32M sucrose to give a final concentration of 2.5% w/v and then homogenized in a glass homogenizer fitted with a teflon pestle, at 850 r.p.m., 10 strokes. The whole homogenate was centrifuged for 10 minutes at 1000*g*. The pellet (crude nuclear fraction) was discarded and the resultant supernatant fluid was thoroughly mixed using a vortex mixer and left on ice until used.

Receptor binding assay

A modified version of the receptor binding assay of Yamamura and Snyder (1974) was employed using tritiated quinuclidinylbenzilate ([^{3}H]QNB) as the radioligand. To assay specific binding of [^{3}H]QNB, 0.2ml (200μl) of the membrane preparation was pre-incubated at 25°C with 2.25ml of 0.05M sodium phosphate buffer, pH 7.4 for 5 min. The pre-incubation period was 15 min when ChE inhibitors were used in order to allow any remaining ACh to be hydrolysed. A 25μl volume of [^{3}H]QNB solution was added together with a 25μl aliquot (giving the required final concentration) of the competing drug and incubated for 60 min. The final concentration of [^{3}H]QNB in each assay vial was 0.9nM ($9.4x10^7$ DPM/ml). After the 60 min incubation, 3ml of the ice-cold sodium phosphate buffer solution were added and the content of the vial was passed through a glass fibre filter (GF/B) positioned in a vacuum-filtration manifold. The filters were washed three times under vacuum with 3ml of the ice-cold sodium phosphate buffer solution. Every determination of binding was done in quadruplicate, together with quadruplicate samples containing 10μM atropine to determine nonspecific [^{3}H]QNB binding. The filters were placed in vials, into which 4ml of the scintillant NE260 was added, left overnight and then counted for tritium using a liquid scintillation counter Beckman LS233, efficiency 35%.

MATERIALS

Chemicals

From Sigma Chemical Co. Ltd, Poole, Dorset, England:

Tacrine hydrochloride hydrate (9-amino-1,2,3,4-tetrahydroacridine), eserine hemisulphate, neostigmine bromide, edrophonium chloride, hemicholinium-3 chloride, reserpine.

From Burroughs Wellcome & Co., London:

Atropine sulphate

Radiochemicals

From Amersham International plc, Buckinghamshire, England:

1) 1-[7,8-^{3}H]Noradrenaline in 0.02M acetic acid:ethanol 9:1 v/v; specific radioactivity 1.48 TBq/mmol (40 Ci/mmol)

2) 5-Hydroxy[G-^{3}H]tryptamine creatinine sulphate in aqueous solution containing 2% ethanol; specific radioactivity 673 GBq/mmol (18.2 Ci/mmol)

3) [7,8-^{3}H]Dopamine in 0.02M acetic acid;ethanol 1:1 v/v; specific radioactivity 1.55TBq/mmol (42Ci/mmol)

4) 1-Quinuclidinyl [phenyl-4-^{3}H]benzilate in ethanol solution; specific radioacivity 1.74 TBq/mmol (47Ci/mmol).

RESULTS

1. The effect of ChE inhibitors on K^+-evoked [^{3}H]ACh release from rat hippocampal prisms.

Tacrine

Shown in Figures 3.1 and 3.2 is the time course of the K^+-stimulated release of radiolabel and the effects of tacrine at the given concentrations in typical experiments. The mean ± SEM S2/S1 ratios in these experiments are 0.632 ± 0.045 and 1.177 ± 0.016 for the controls and 30μM tacrine respectively in Figure 3.1 and 0.712 ± 0.047, 0.473 ± 0.062 and 0.196 ± 0.005 for controls, 100μM and 200μM tacrine respectively, in Figure 3.2.

CONCENTRATION TACRINE	S2/S1 RATIO	
	CONTROL	TACRINE
10μM	0.698 ± 0.024 (n=14)	0.891 ± 0.039* (n=14)
30μM	0.671 ± 0.027 (n=16)	1.138 ± 0.056** (n=19)
50μM	0.698 ± 0.024 (n=14)	0.921 ± 0.043* (n=16)
100μM	0.713 ± 0.034 (N=4)	0.562 ± 0.086* (N=4)
200μM	0.713 ± 0.034 (N=4)	0.245 ± 0.033** (N=4)

TABLE 3.1

The effect of tacrine on K^+-evoked [^{3}H]ACh release. The test samples were superfused with tacrine 10 min prior to and during the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of the means (N) or the mean ± SEM of the total number of replicate samples (n) observed from four experiments, depending on the analysis of variance; see Methods (Chapter 2) for details. Significant differences with respect to the controls are denoted by * $P<0.001$ and ** $P<0.0001$.

As shown in Figure 3.3 and Table 3.1 above, tacrine in the concentration range of 10-50μM significantly increased the S2/S1 ratio of evoked [^{3}H]ACh release. The maximal effect was observed at 30μM tacrine which produced a 70% increase of

[^{3}H]ACh release above control levels. The concentrations of tacrine above 50μM tested in this study, produced a marked reduction in [^{3}H]ACh release. At 100μM and 200μM tacrine, release was inhibited by 21% ($P<0.001$) and 66% ($P<0.0001$) of the control value. These results are shown in the bell-shaped concentration-effect curve in Figure 3.3. Tacrine did not alter the basal release of [^{3}H]ACh at the concentrations tested (Figures 3.1 & 3.2).

Eserine

A typical effect of 100μM eserine on K^+-stimulated [^{3}H]ACh release compared with the controls is shown in Figure 3.4. The mean ± SEM S2/S1 ratios corresponding to the data plotted in Figure 3.4 are 0.727 ± 0.013 and 0.997 ± 0.065 for controls and 100μM eserine respectively.

The S2/S1 ratios in the presence of eserine at concentrations ranging from 10-200μM are shown in Figure 3.5 and Table 3.2 below. Although all of the four concentrations tested increased [^{3}H]ACh release significantly, a concentration-

CONCENTRATION ESERINE	S2/S1 RATIO	
	CONTROL	ESERINE
10μM	0.734 ± 0.030 (n=15)	0.965 ± 0.039* (n=15)
30μM	0.734 ± 0.030 (n=15)	0.997 ± 0.026** (n=16)
100μM	0.725 ± 0.029 (n=13)	1.042 ± 0.039** (n=15)
200μM	0.725 ± 0.029 (n=13)	1.026 ± 0.036** (n=16)

TABLE 3.2

The effect of eserine on K^+-evoked [^{3}H]ACh release. The test samples were superfused with eserine 10 min prior to and during the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of the total number of replicate samples (n) observed from four experiments; see Methods (Chapter 2) for details. Significant differences with respect to the controls are denoted by * $P<0.001$ and **$P<0.0001$.

related effect is not apparent. Eserine at 10μM concentrations showed a 36% increase in S2/S1 ratio whereas at 100μM and 200μM, a 44% and 42% increase was observed. Eserine did not affect the basal release of [^{3}H]ACh at any of the concentrations tested.

Neostigmine.

Typical effects of neostigmine at concentrations of 30 and 100μM on K^+-stimulated [^{3}H]ACh release are shown in Figure 3.6. The corresponding mean ± SEM of the S2/S1 ratios are 0.673 ± 0.037, 0.903 ± 0.069 and 1.393 ± 0.121 for the control, 30μM and 100μM neostigmine groups respectively.

The effect of neostigmine in the concentration range 10-200μM is shown in Figure 3.7 and Table 3.3 below. Neostigmine significantly increased release in a concentration-dependent manner with a maximal effect at 100μM. At this concentration neostigmine produced an increase of 115%, and also at the lowest concentration of 10μM, an increase of 20%, which was still statistically significant.

CONCENTRATION NEOSTIGMINE	S2/S1 RATIO	
	CONTROL	NEOSTIGMINE
10μM	0.734 ± 0.029 (n=14)	0.882 ± 0.026* (n=16)
30μM	0.653 ± 0.024 (n=13)	1.082 ± 0.083* (n=16)
100μM	0.653 ± 0.024 (n=13)	1.405 ± 0.079** (n-16)
200μM	0.734 ± 0.029 (n=14)	1.133 ± 0.059** (n=14)

TABLE 3.3

The effect of neostigmine on K^+-evoked [^{3}H]ACh release. The test samples were superfused with neostigmine 10 min prior to and during the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of the total number of replicate samples (n) observed from four experiments; see Methods (Chapter 2) for details. Significant differences with respect to the controls are denoted by

* P<0.001 and ** P<0.0001.

Neostigmine did not affect the basal release of [^{3}H]ACh at any of the concentrations tested.

Figure 3.7 shows an apparent decrease in [^{3}H]ACh release at 200μM concentrations of neostigmine. However, since the effects of 100 and 200μM neostigmine were not compared within the same experiment, the relative effects of 100 and 200μM concentrations were compared in a further group of experiments. As shown in Figure 3.8a, no difference in the S2/S1 ratio was observed at 100 and 200μM neostigmine (control S2/S1 0.706 ± 0.042 (n=15), 100μM neostigmine S2/S1 1.607 ± 0.086 (n=15), 200μM neostigmine S2/S1 1.513 ± 0.076 (n=16). Figure 3.8b also shows the effect of superfusing the prisms with neostigmine only during the S2 stimulation rather than 10 minutes prior to as well as during S2 stimulation. The S2/S1 ratio was slightly greater when neostigmine was present only during the S2 stimulation - S2/S1 (S2 only) 1.171 ± 0.047 (n=7) compared with 1.019 ± 0.050 (n=8); $P<0.05$.

Edrophonium

CONCENTRATION EDROPHONIUM	S2/S1 RATIO	
	CONTROL	EDROPHONIUM
10μM	0.601 ± 0.027 (n=15)	0.799 ± 0.042** (n=16)
30μM	0.601 ± 0.027 (n=15)	0.829 ± 0.037** (n=15)
100μM	0.627 ± 0.063 (N=4)	1.00 ± 0.074* (N=4)
200μM	0.627 ± 0.063 (N=4)	1.13 ± 0.071** (N=4)

TABLE 3.4

The effect of edrophonium on K^+-evoked [^{3}H]ACh release. The test samples were superfused with edrophonium 10 min prior to and during the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of the means (N) or the mean ± SEM of the total number of replicate samples (n) observed from four experiments, depending on the analysis of variance; see Methods (Chapter 2) for details. Significant differences with respect to the controls are denoted by * $P<0.05$ and ** $P<0.01$.

Figure 3.9 shows the profile of K^+-stimulated release and the typical effect of 100μM edrophonium. The mean ± SEM S2/S1 ratios corresponding to the data shown in Figure 3.9 are 0.798 ± 0.029 and 1.304 ± 0.103 for controls and 100μM edrophonium respectively.

Edrophonium increases [^{3}H]ACh release and the S2/S1 ratios (Figure 3.10 and Table 3.4) in a concentration-dependent manner in the range 10-200μM. At the lowest concentration of 10μM edrophonium, there was a 33% increase and at 200μM an 81% increase was observed. The S2/S1 ratios for 100μM and 200μM edrophonium are not significantly different from each other ($P>0.05$) and therefore these concentrations appear to be maximally effective. Edrophonium did not affect basal release of [^{3}H]ACh at any of the concentrations tested.

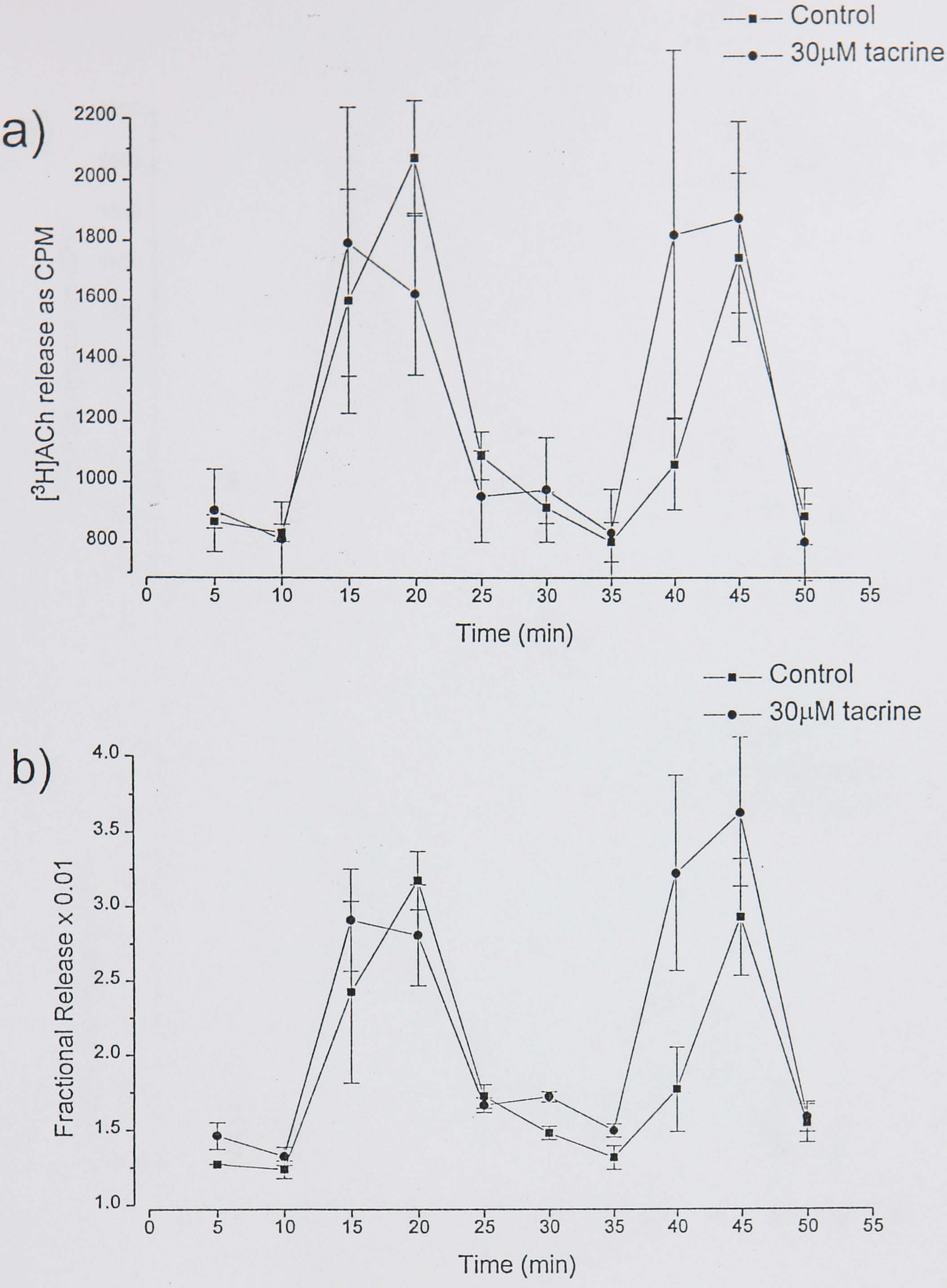


Figure 3.1 The release of radiolabel from rat hippocampal prisms evoked by 30mM K^+ - the effect of tacrine (30μM).

The prisms were preloaded with [^{3}H]choline (see Chapter 2 for experimental details) and stimulated twice, (S1 and S2) for 2 min. Tacrine was added to the superfusion medium 10 min prior to and during the S2 stimulation, in the test group. Each point represents release as the mean ± SEM of the replicate samples (per group) from one typical experiment expressed as:

a) CPM

b) fractional release

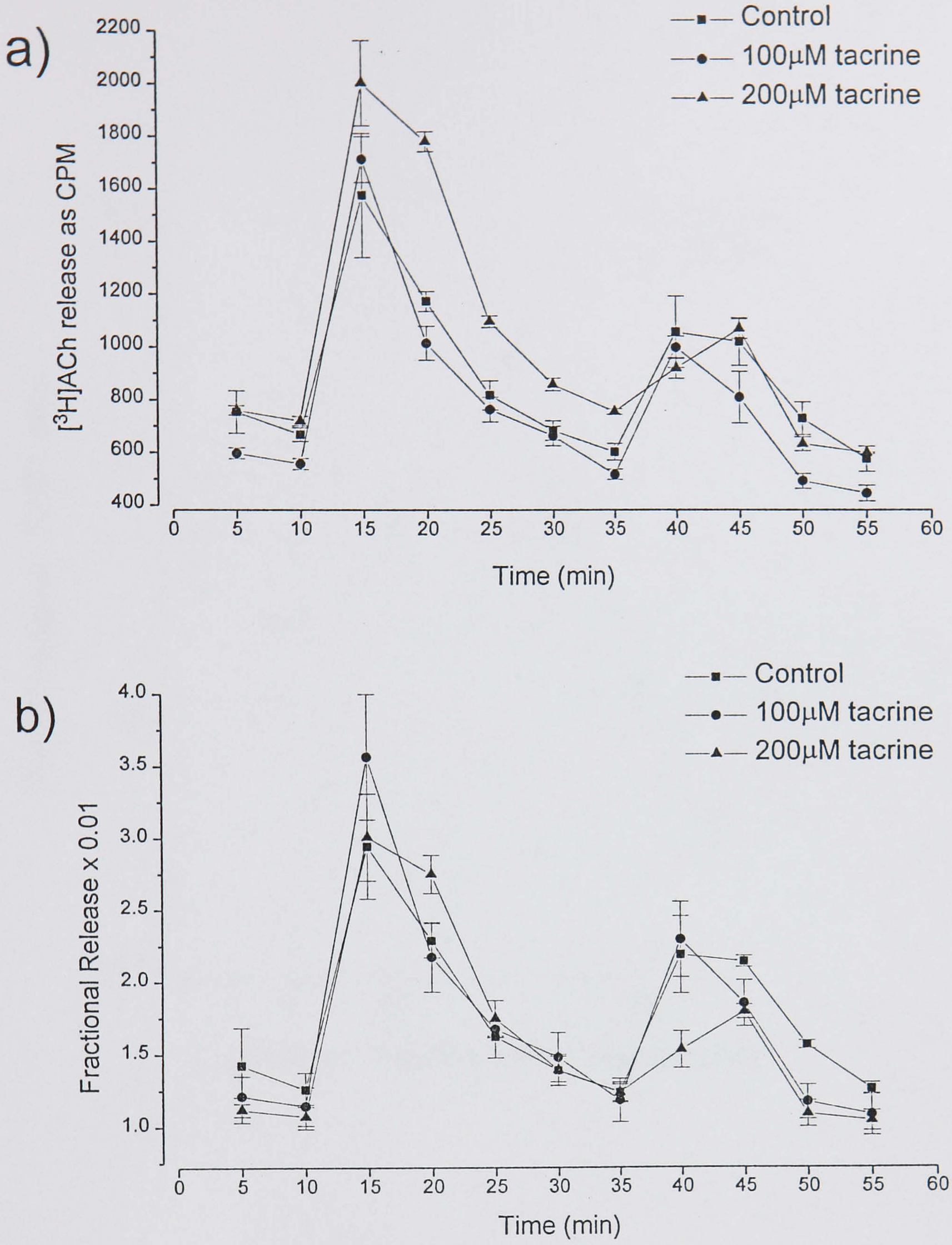


Figure 3.2 The release of radiolabel from rat hippocampal prisms evoked by 30mM K^+ - the effect of tacrine (100, 200μM).

The prisms were preloaded with [^{3}H]choline (see Chapter 2 for experimental details) and stimulated twice, (S1 and S2) for 2 min. Tacrine was added to the superfusion medium 10 min prior to and during the S2 stimulation, in the test group. Each point represents release as the mean ± SEM of the replicate samples (per group) from one typical experiment, expressed as:

a) CPM

b) fractional release.

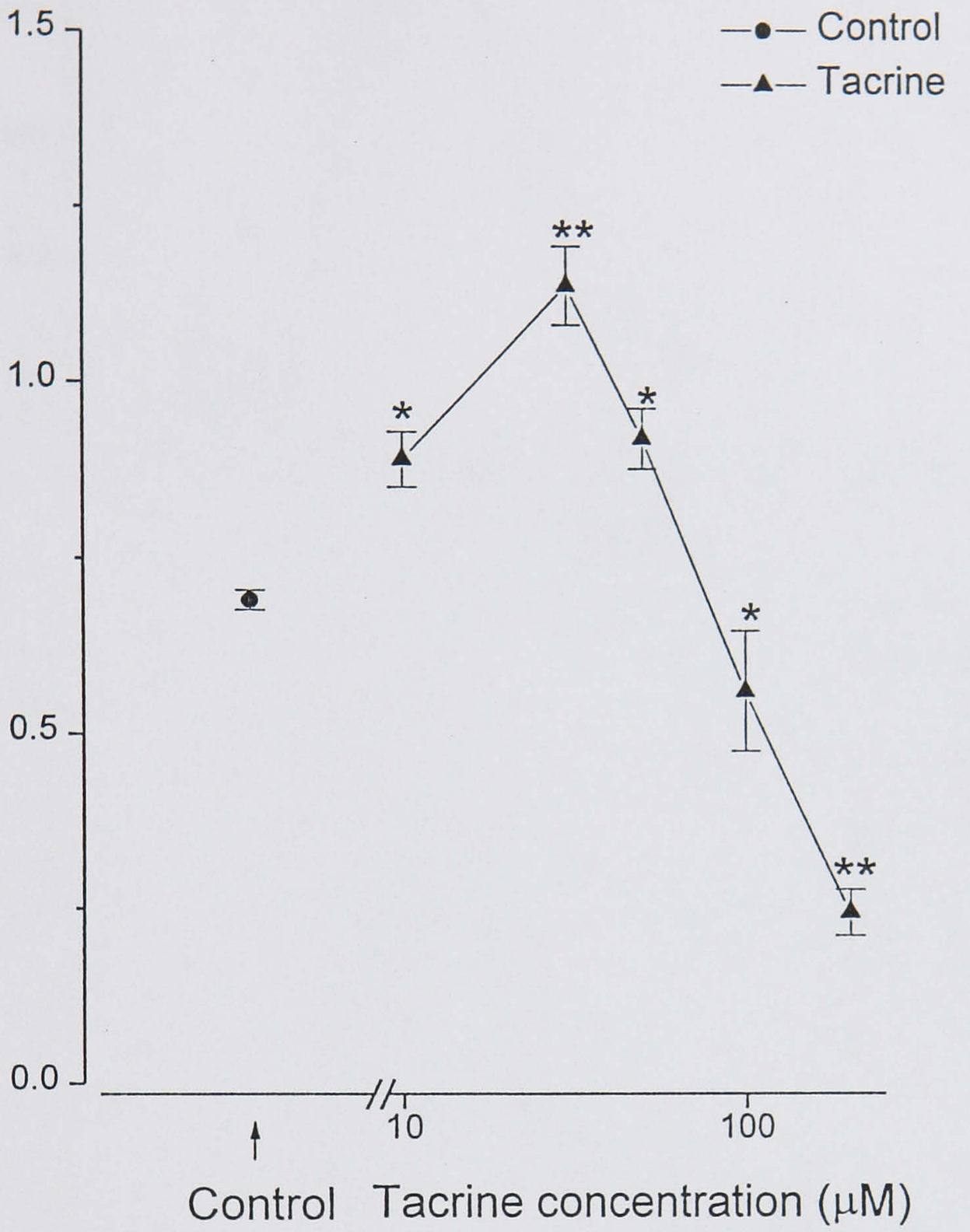
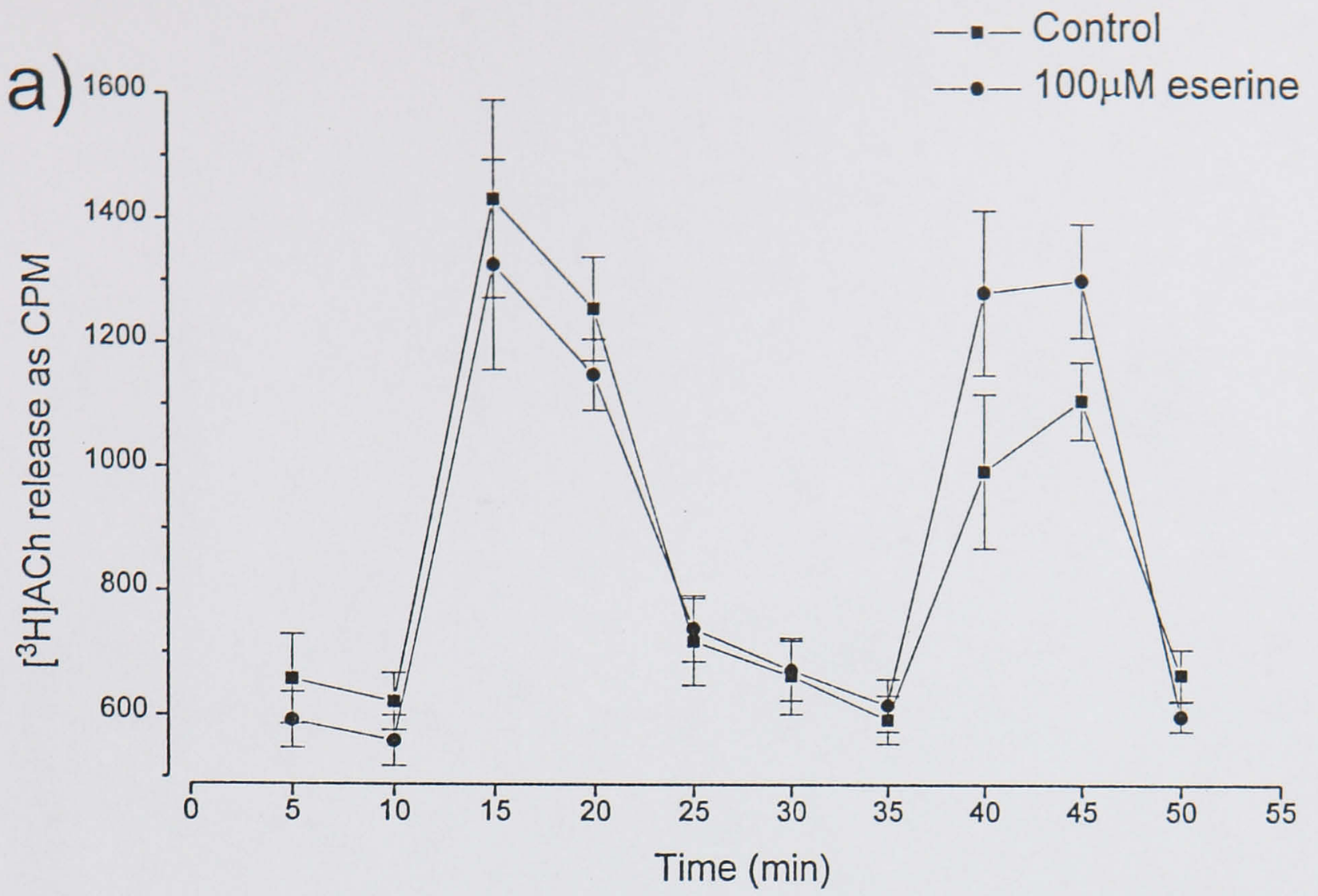


Figure 3.3 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippcampal prisms - the effect of tacrine (10-200μM).

The prisms were stimulated twice (S1 and S2) for 2 min and tacrine was added to the superfusion medium 10 min prior to and during the S2 stimulation. Each point represents the S2/S1 ratio as the mean ± SEM of the means (N) or the mean ± SEM of the total number of replicate samples (n) observed from 4 experiments; see Table 3.1 for details. The control represents the mean ± SEM of means obtained from all the experiments. Significant differences in the S2/S1 ratio compared to the control group are denoted by *P<0.001 and ** P<0.0001.



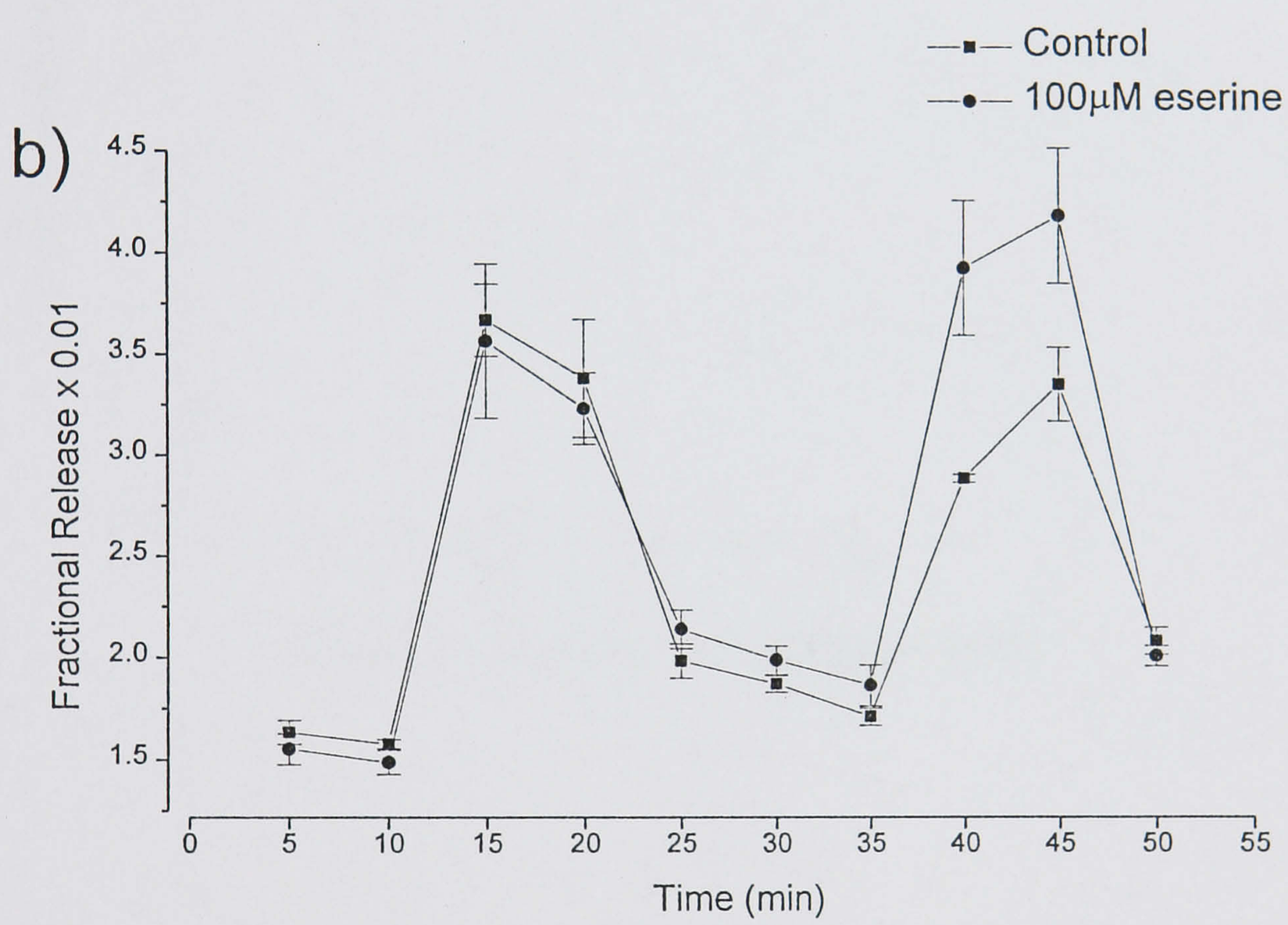


Figure 3.4 The release of radiolabel from rat hippocampal prisms evoked by 30mM K^+ - the effect of eserine.

The prisms were preloaded with [^{3}H]choline (see Chapter 2 for experimental details) and stimulated twice, (S1 and S2) for 2 min. Eserine was added to the superfusion medium 10 min prior to and during the S2 stimulation, in the test group. Each point represents release as the mean ± SEM of the replicate samples (per group) from one typical experiment, expressed as:

a) CPM

b) fractional release.

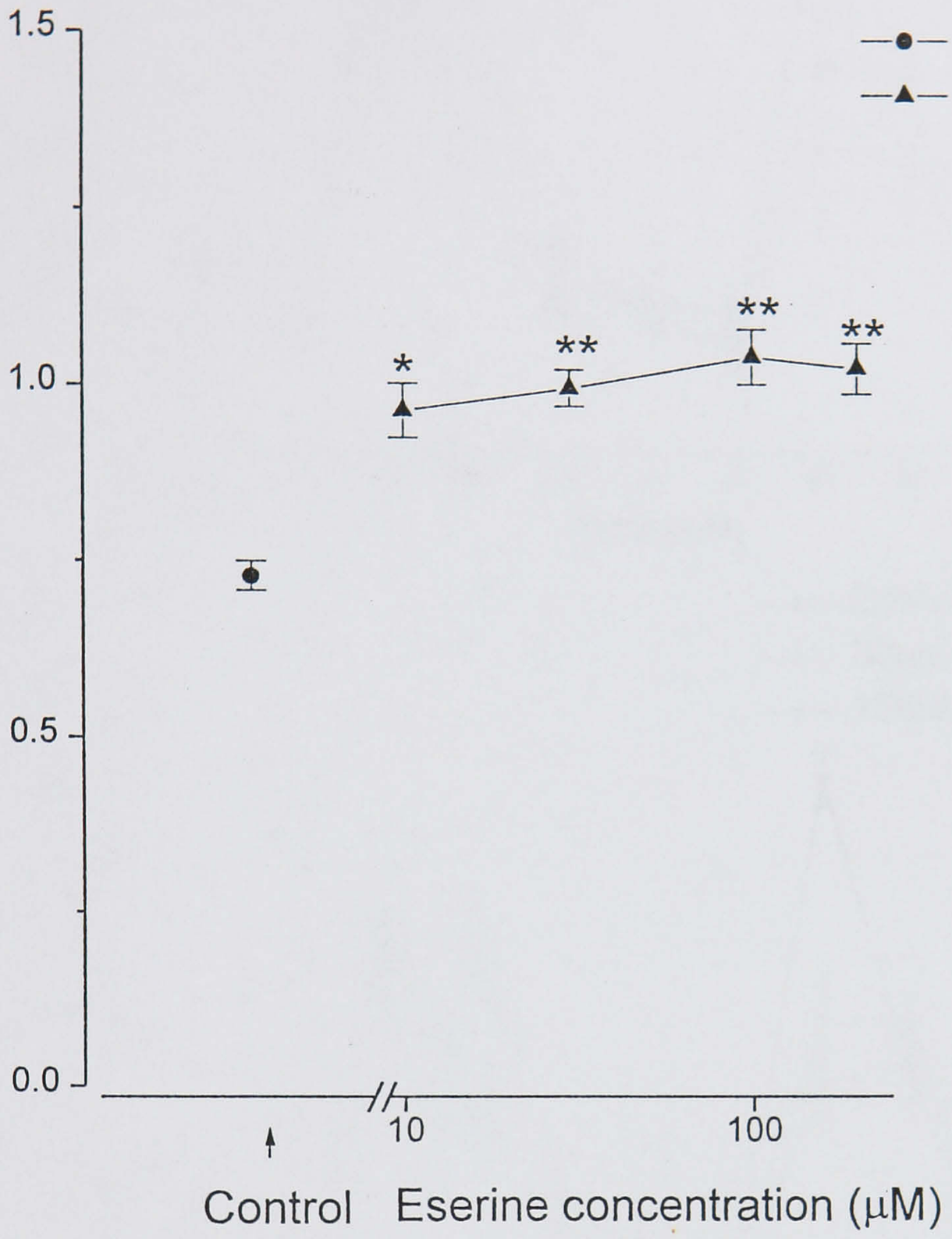
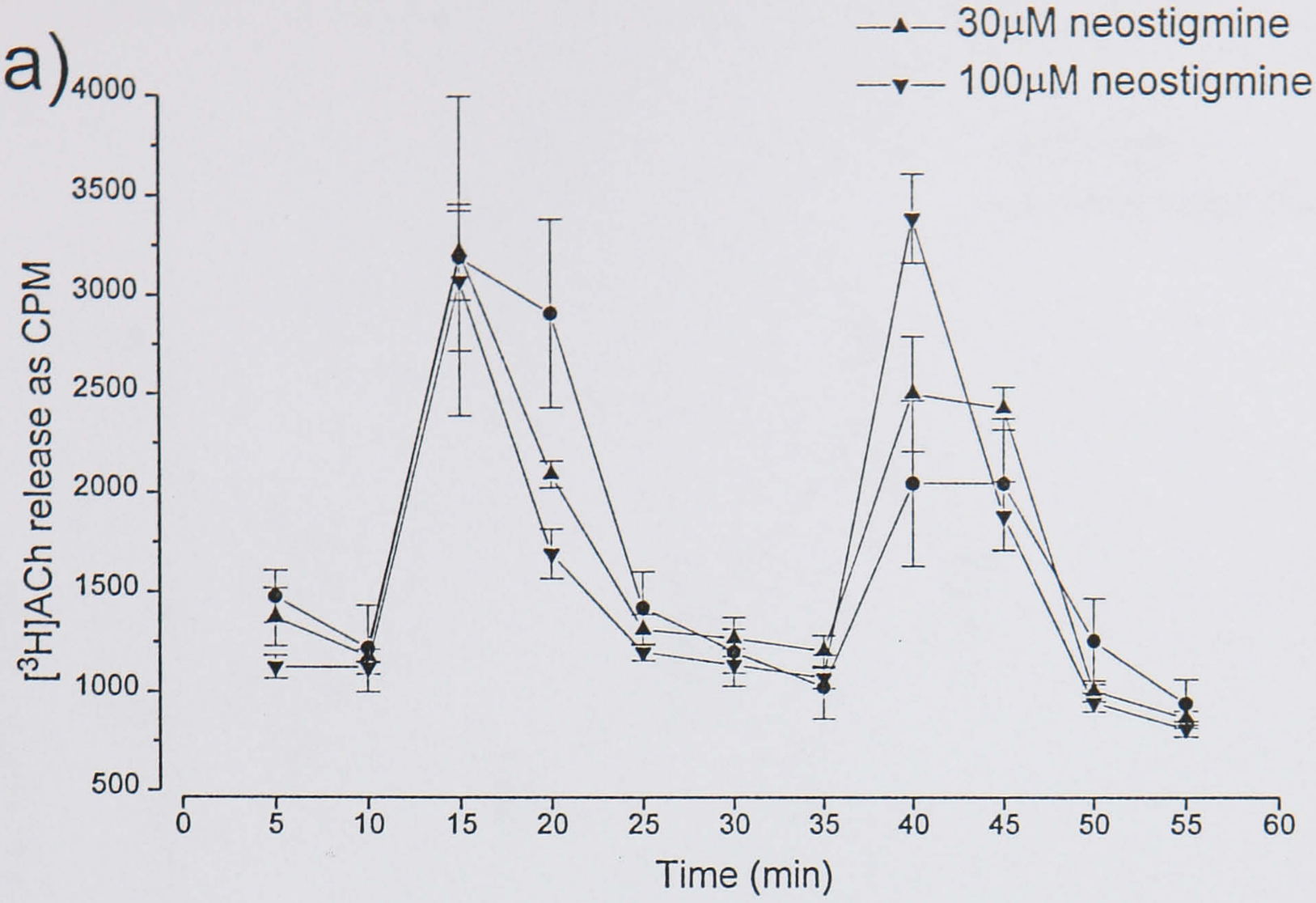


Figure 3.5 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippcampal prisms - the effect of eserine (10-200μM);

The prisms were stimulated twice (S1 and S2) for 2 min and eserine was added to the superfusion medium 10 min prior to and during the S2 stimulation. Each point represents the S2/S1 ratio as the mean ± SEM of the total number of replicate samples (n) observed from 4 experiments; see Table 3.2 for details. The control represents the mean ± SEM of all the replicate samples obtained from all the experiments. Significant differences in the S2/S1 ratio compared to the control group are denoted by *P<0.001 and ** P<0.0001.



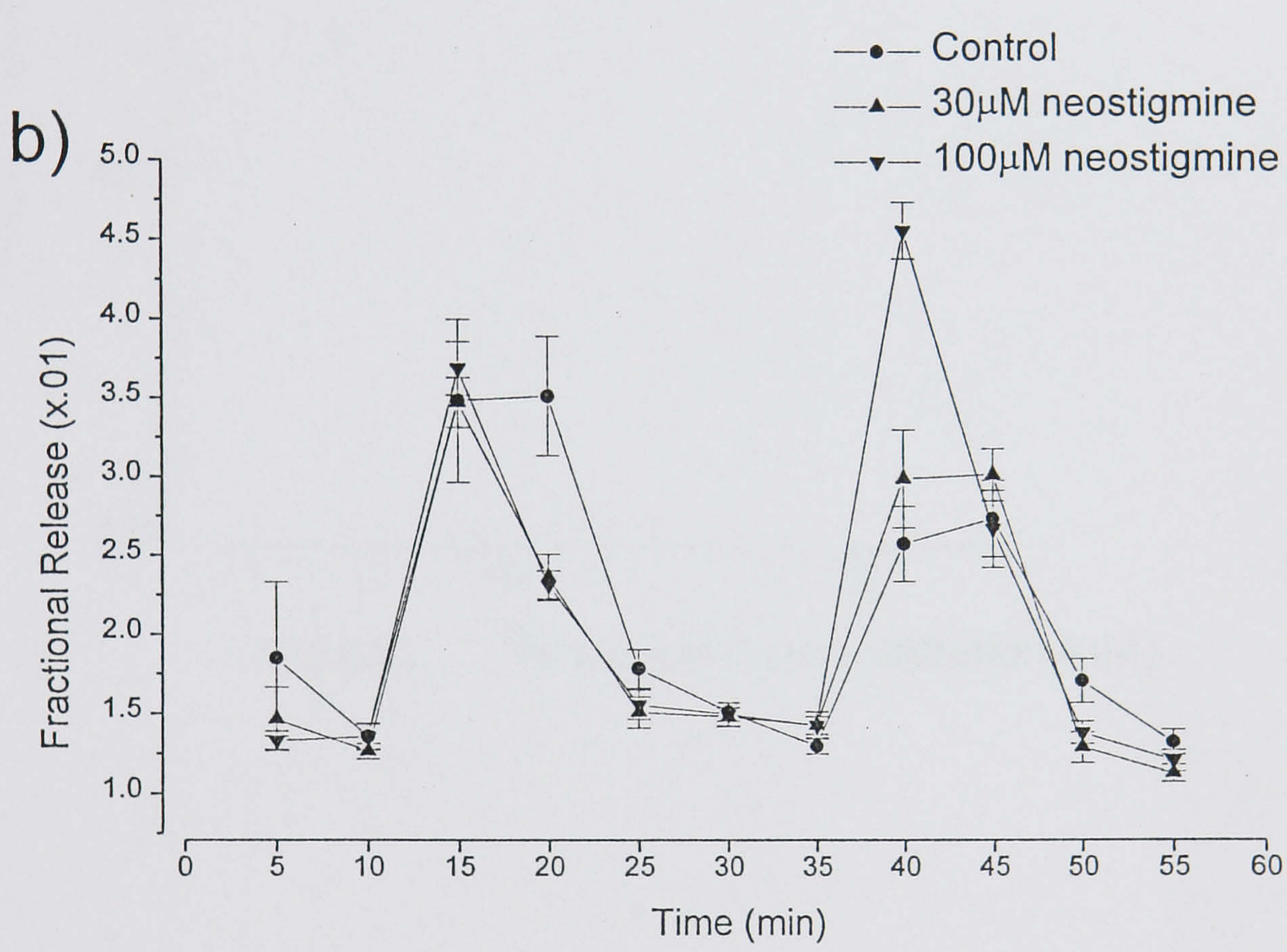


Figure 3.6 The release of radiolabel from rat hippocampal prisms evoked by 30mM K^+ - the effect of neostigmine.

The prisms were preloaded with [^{3}H]choline (see Chapter 2 for experimental details) and stimulated twice, (S1 and S2) for 2 min. Neostigmine was added to the superfusion medium 10 min prior to and during the S2 stimulation, in the test group. Each point represents release as the mean ± SEM of the replicate samples (per group) from one typical experiment, expressed as:

a) CPM

b) fractional release.

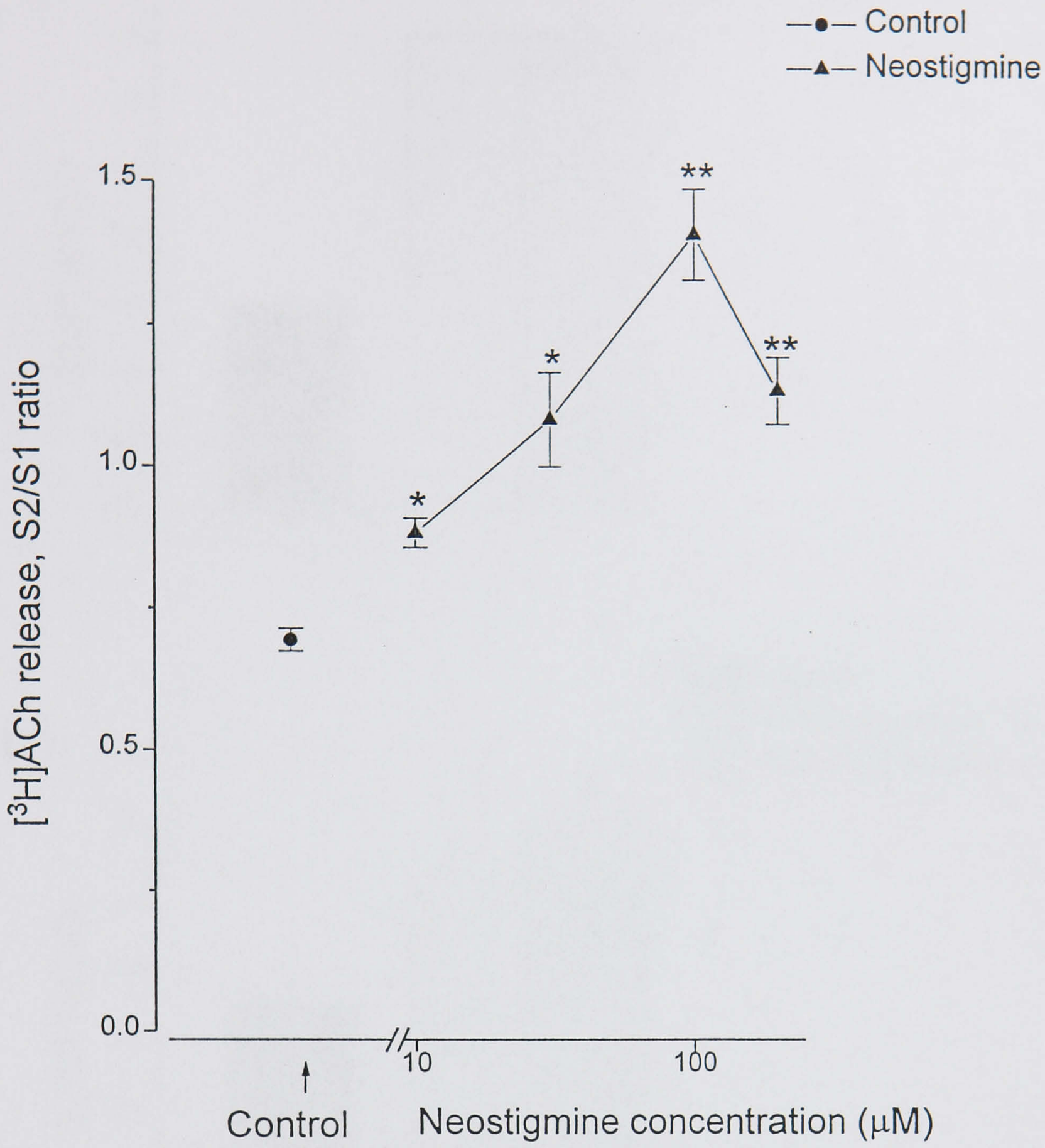


Figure 3.7 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippcampal prisms - the effect of neostigmine (10-200μM).

The prisms were stimulated twice (S1 and S2) for 2 min and neostigmine was added to the superfusion medium 10 min prior to and during the S2 stimulation. Each point represents the S2/S1 ratio as the mean ± SEM of the total number of replicate samples (n) observed from 4 experiments; see Table 3.3 for details. The control represents the mean ± SEM of means from all the experiments. Significant differences in the S2/S1 ratio compared to the control group are denoted by *P<0.001 and ** P<0.0001.

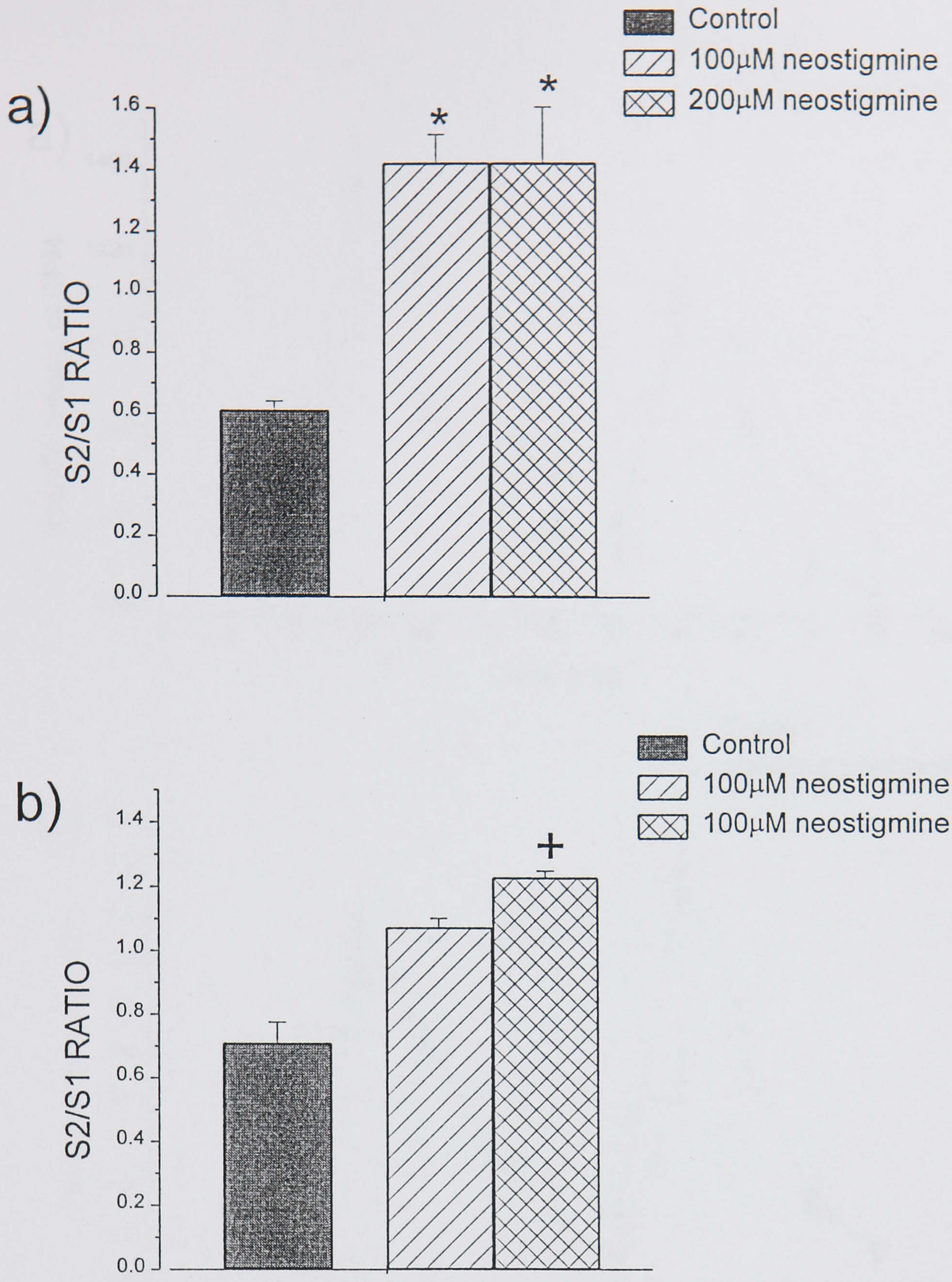


Figure 3.8 Comparison of the effects of neostigmine on the S2/S1 ratio for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms.

a) 100 and 200μM neostigmine, 10 min prior to and during the 2 min S2 stimulation

b) comparison of the effect of neostigmine on the S2 release in 12 and 2 minute treatments.

Each column represents the S2/S1 ratio as the mean ± SEM of the total number of replicate samples (n) obtained from 3 (a) and 2 (b) experiments. Significant differences in the S2/S1 ratios compared to a) control group b) 100μM neostigmine added 10 min prior to and during the 2 min S2 stimulation, are denoted by * $P<0.001$ and † $P<0.05$.

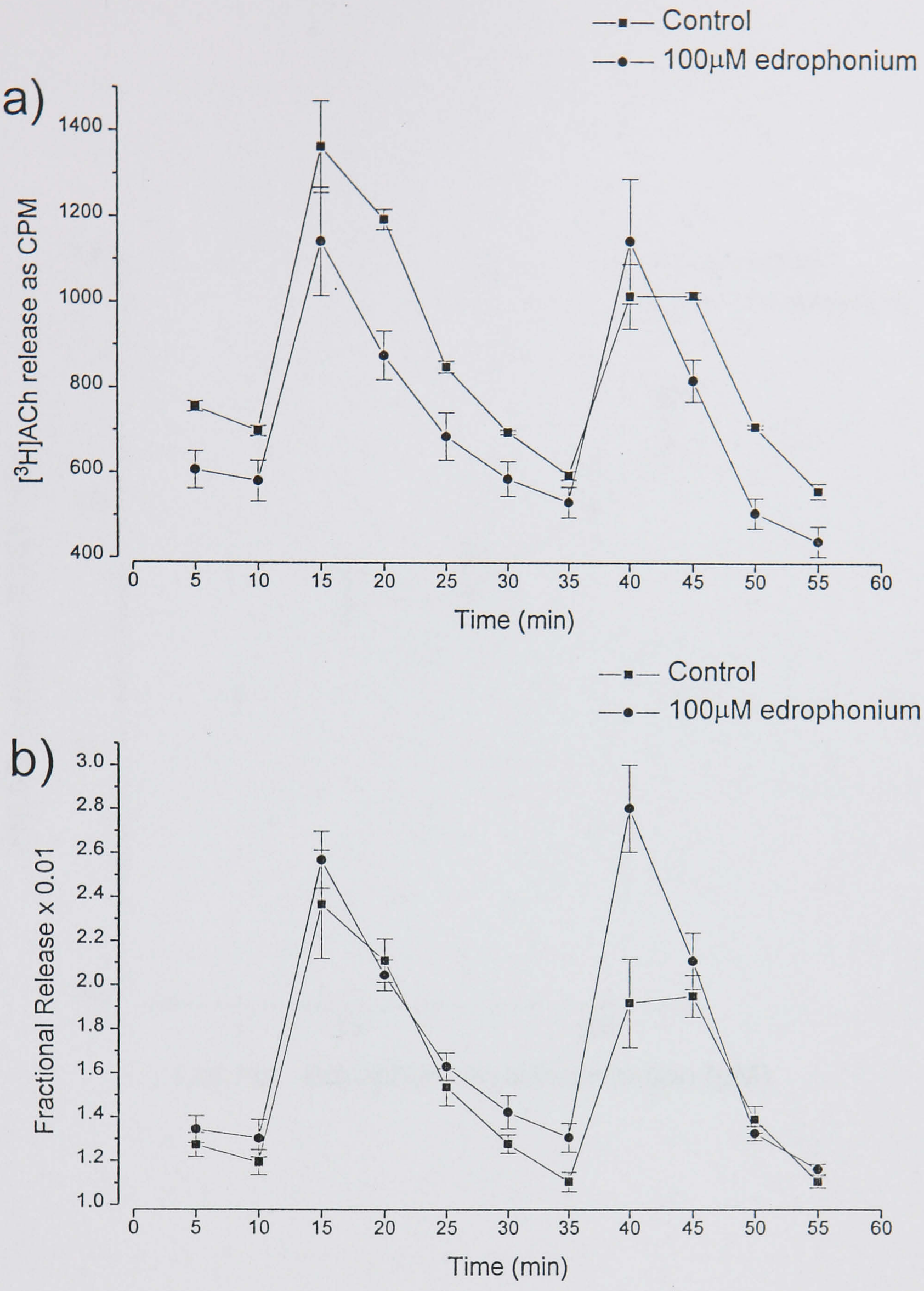


Figure 3.9 The release of radiolabel from rat hippocampal prisms evoked by 30mM K^+ - the effect of edrophonium.

The prisms were preloaded with [^{3}H]choline (see Chapter 2 for experimental details) and stimulated twice, (S1 and S2) for 2 min. Edrophonium was added to the superfusion medium 10 min prior to and during the S2 stimulation, in the test group. Each point represents release as the mean ± SEM of the replicate samples (per group) from one typical experiment, expressed as:

a) CPM

b) fractional release.

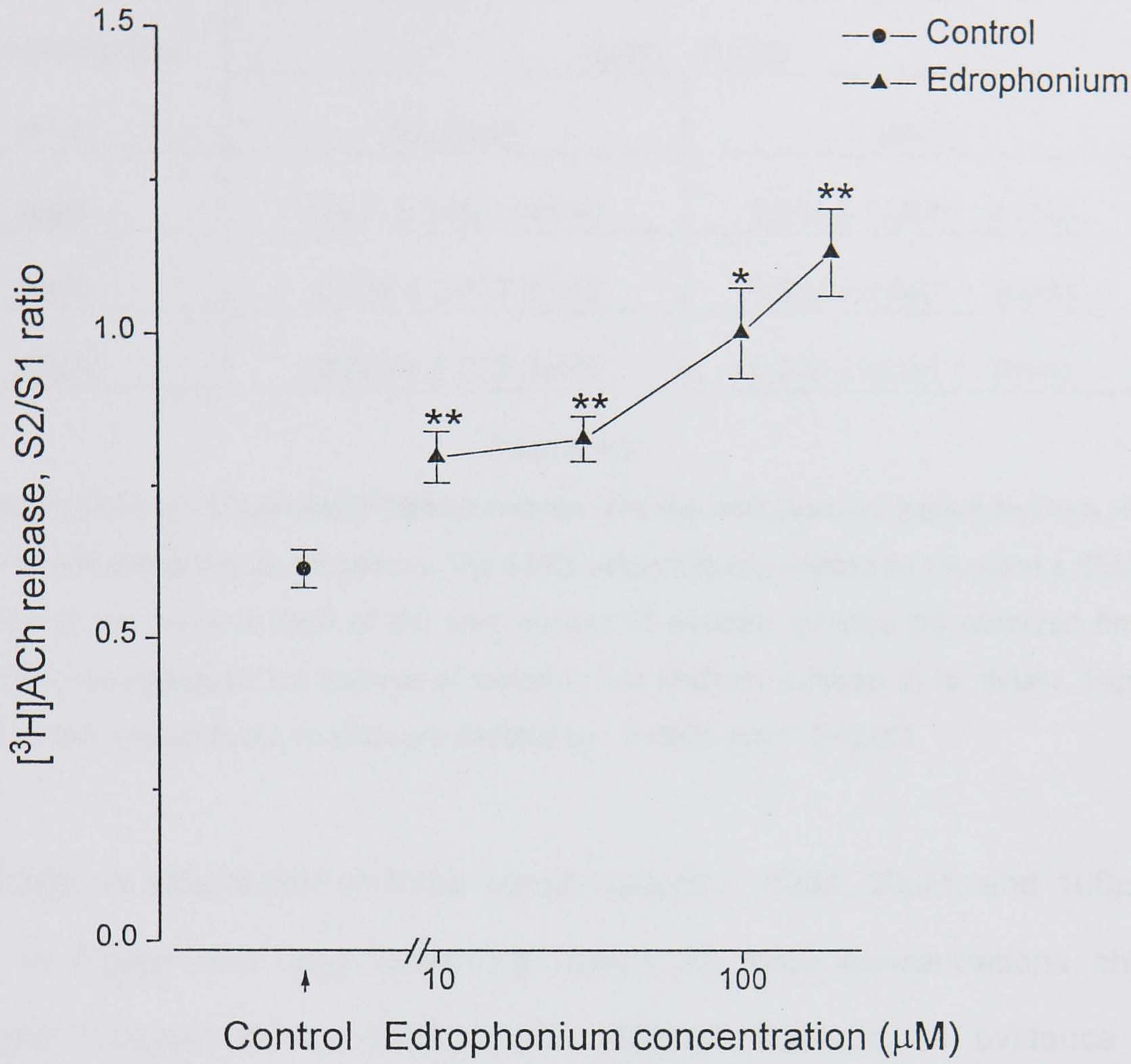


Figure 3.10 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippcampal prisms - the effect of edrophonium (10-200μM).

The prisms were stimulated twice (S1 and S2) for 2 min and edrophonium was added to the superfusion medium 10 min prior to and during the S2 stimulation. Each point represents the S2/S1 ratio as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) observed from 4 experiments; see Table 3.4 for details. The control represents the mean ± SEM of means from all the experiments. Significant differences in the S2/S1 ratio compared to the control group are denoted by *P<0.05 and ** P<0.01.

2. The effect of hemicholinium-3, a choline uptake blocker on K^+-evoked [^{3}H]ACh release from rat hippocampal prisms.

CONCENTRATION HC-3	S2/S1 RATIO	
	CONTROL	HC-3
10μM	0.711 ± 0.024 (n=14)	0.897 ± 0.032** (n=16)
30μM	0.729 ± 0.022 (N=4)	0.820 ± 0.040* (N=4)
100μM	0.729 ± 0.022 (N=4)	0.990 ± 0.084** (N=4)

TABLE 3.5

The effect of HC-3 on K^+-evoked [^{3}H]ACh release. The test samples were superfused with HC-3 10 min prior to and during the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of the means (N) or the mean ± SEM of the total number of replicate samples (n) observed from four experiments, depending on the analysis of variance; see Methods (Chapter 2) for details. Significant differences with respect to the controls are denoted by * $P<0.05$ and ** $P<0.001$.

Hemicholinium was tested at three concentrations - 10μM, 30μM, and 100μM as shown in Figure 3.11 and Table 3.5 above. All three concentrations show a significant increase in the S2/S1 ratio, although there is no evidence of a concentration-dependent effect. Concentrations 10μM, 30μM and 100μM showed increases of 26%, 13% and 36% and no significant difference was observed between 30μM (13%) and 100μM (36%) HC-3 ($P>0.05$).

3. Combination of the effects of hemicholinium and ChE inhibitors on K^+-stimulated [^{3}H]ACh release from rat hippocampal prisms.

In the following Figures, significant differences are indicated with respect to release in the presence of HC-3 (100μM) or the ChE inhibitors alone, although these are statistically different from the controls as demonstrated previously (Tables 3.1-3.5 and Figures 3.3, 3.5, 3.7, 3.10 and 3.11).

Tacrine and HC-3

As shown in Figure 3.12a, the effect of the combination of 30μM tacrine and 100μM HC-3 together is significantly greater ($P<0.001$) than that of 100μM HC-3 alone. However when compared with 30μM tacrine alone as in Figure 3.12b, the effect of the combination was not significantly different; $P>0.05$. See also Table 3.6.

Eserine and HC-3

Similar results for eserine are shown in Figures 3.13a and 3.13b. The S2/S1 ratios for 100μM HC-3 alone compared with 100μM HC-3 and 100μM eserine together are significantly different ($P<0.05$) whereas no difference was observed between 100μM eserine alone and 100μM HC3 and 100μM eserine together ($P>0.05$). See also Table 3.6.

Neostigmine and HC-3

Neostigmine (100μM) was also shown to have a significant effect on [^{3}H]ACh release over and above the effect of 100μM HC-3 as shown in Figure 3.14a ($P<0.001$) although 100μM HC-3 was not seen to have an effect additional to that of 100μM neostigmine ($P>0.05$) (Figure 3.14b). See also Table 3.6.

Edrophonium and HC-3

Similar to the ChE inhibitors mentioned above, the effect of 100μM edrophonium together with 100μM HC-3 was shown (Figure 3.15a) to be significantly greater than 100μM HC-3 alone ($P<0.0001$) although not significantly different when compared with 100μM edrophonium alone as shown in Figure 3.15b ($P>0.05$). See also Table 3.6.

CHE-INHIBITOR (CHE-I)	S2/S1 RATIO		
	CHE-INHIBITOR	HC-3	CHE-INHIBITOR + HC-3
TACRINE (30μM)	0.997 ± 0.079 (N=4) _	_ 0.807 ± 0.026 (N=4)	1.096 ± 0.056 (N=4) 1.158 ± 0.082*** (N=4)
ESERINE (100μM)	1.124 ± 0.072 (N=4) _	_ 0.921 ± 0.065 (N=4)	1.205 ± 0.031 (N=4) 1.355 ± 0.089* (N=4)
NEOSTIGMINE (100μM)	1.269 ± 0.051 (n=14) _	_ 1.026 ± 0.059 (n=14)	1.429 ± 0.070 (n=15) 1.415 ± 0.064*** (n=15)
EDROPHONIUM (100μM)	1.280 ± 0.035 (n=14) _	_ 0.934 ± 0.043 (n=25)	1.364 ± 0.048 (n=16) 1.267 ± 0.046**** (n=25)

TABLE 3.6

Comparison of the effects of ChE-inhibitor and HC-3, individually and in combination. The test samples were superfused with ChE-inhibitor, HC-3 or ChE-inhibitor and HC-3 together, 10 min prior to and during the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) observed from 4-5 experiments, depending on the analysis of variance; see Methods (Chapter 2) for details. Significant differences with respect to HC-3 are denoted by * P<0.05, *** P<0.001, **** P<0.0001.

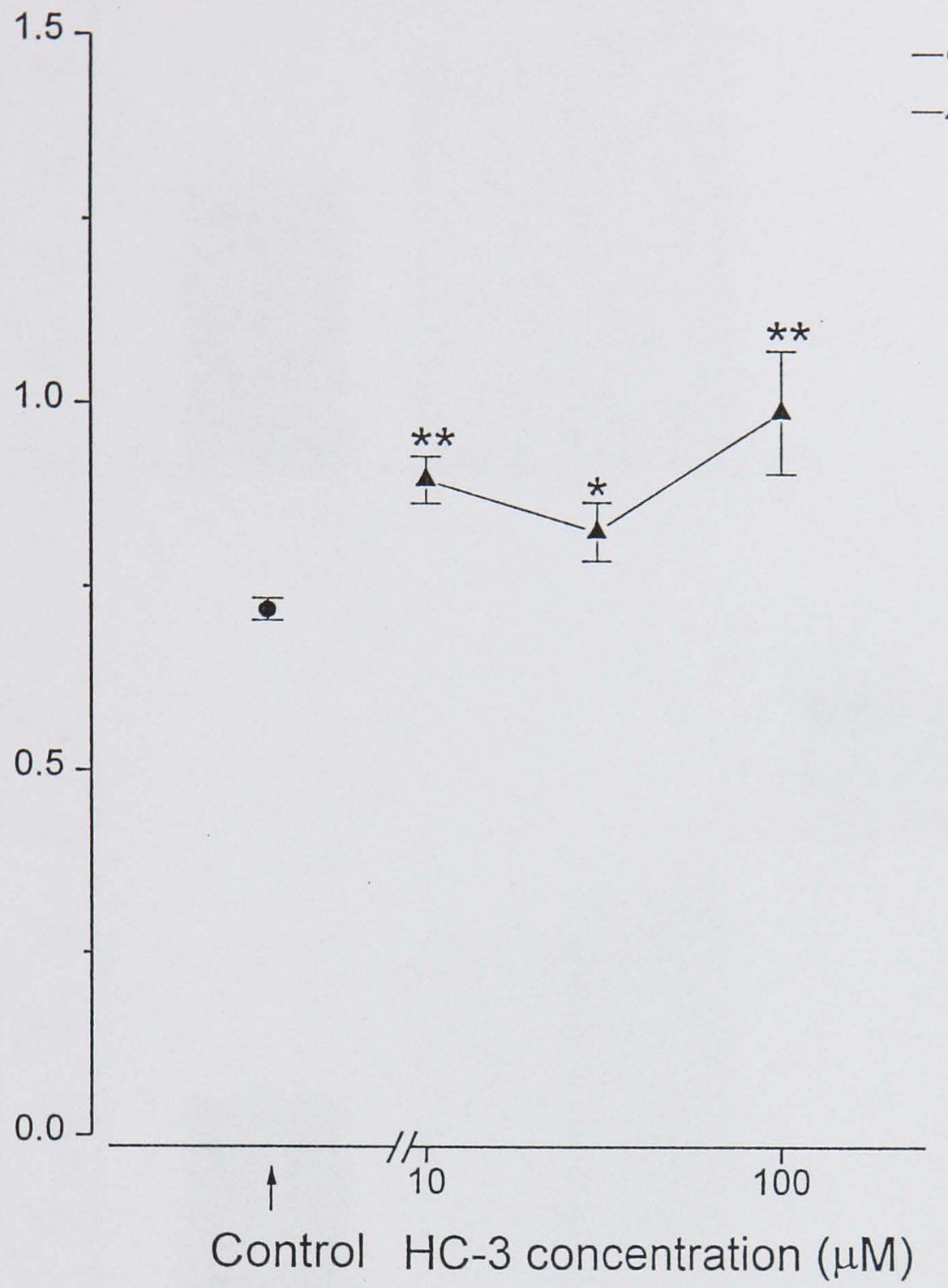


Figure 3.11 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippcampal prisms - the effect of hemicholinium (HC-3) (10-100μM).

The prisms were stimulated twice (S1 and S2) for 2 min and HC-3 was added to the superfusion medium 10 min prior to and during the S2 stimulation. Each point represents the S2/S1 ratio as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) observed from 4 experiments; see Table 3.5 for details. The control represents the mean ± SEM of means from all the experiments. Significant differences in the S2/S1 ratio compared to the control group are denoted by *P<0.05 and ** P<0.001.

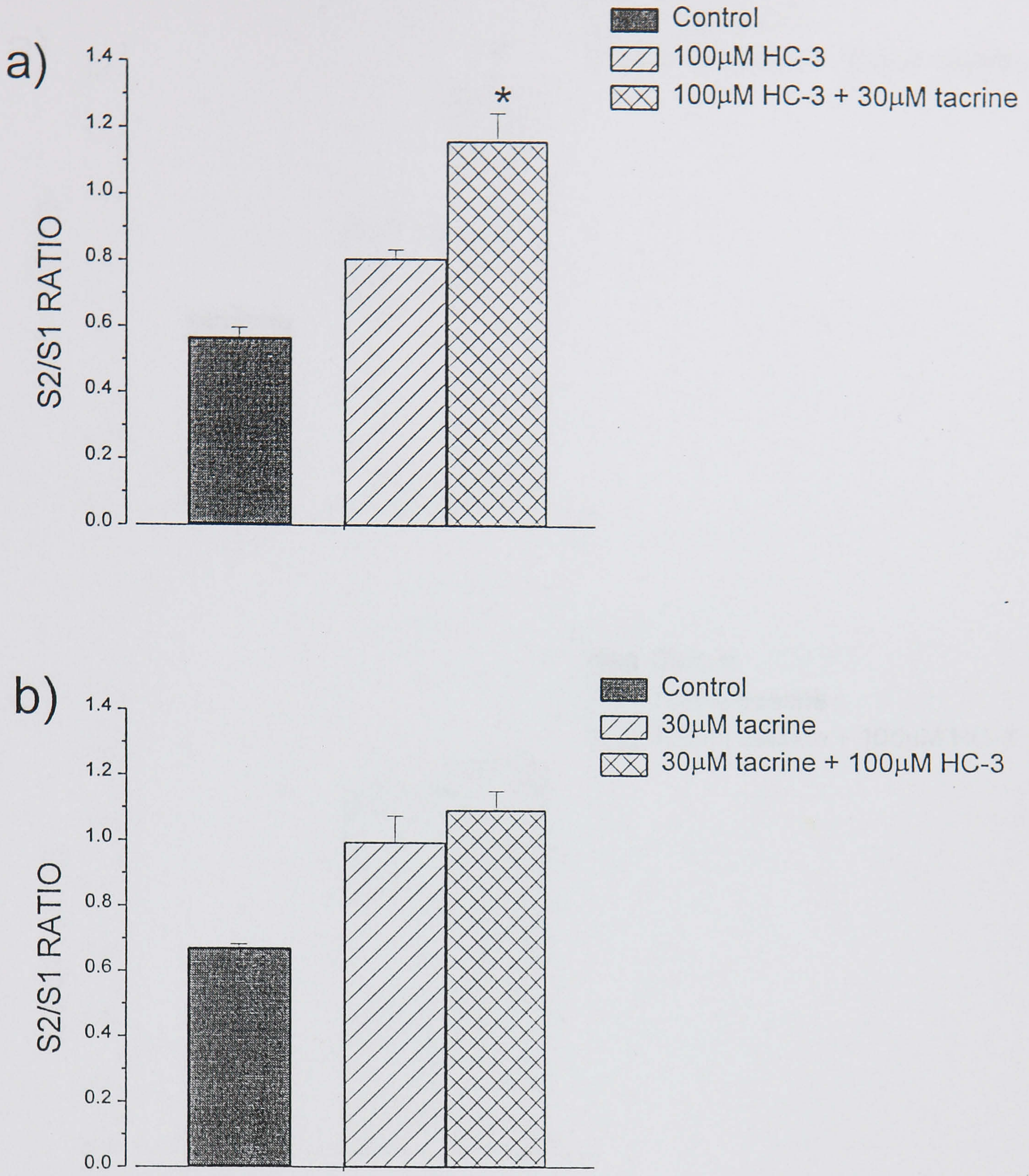


Figure 3.12 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effects of tacrine and HC-3, individually and in combination.

a) HC-3 compared with HC-3 and tacrine together

b) tacrine compared with HC-3 and tacrine together

Each column represents the S2/S1 ratio as the mean ± SEM of means (N) obtained from 4 experiments; see Table 3.6 for details. Significant differences in the S2/S1 ratio compared to HC-3 or tacrine alone are denoted by * P< 0.001.

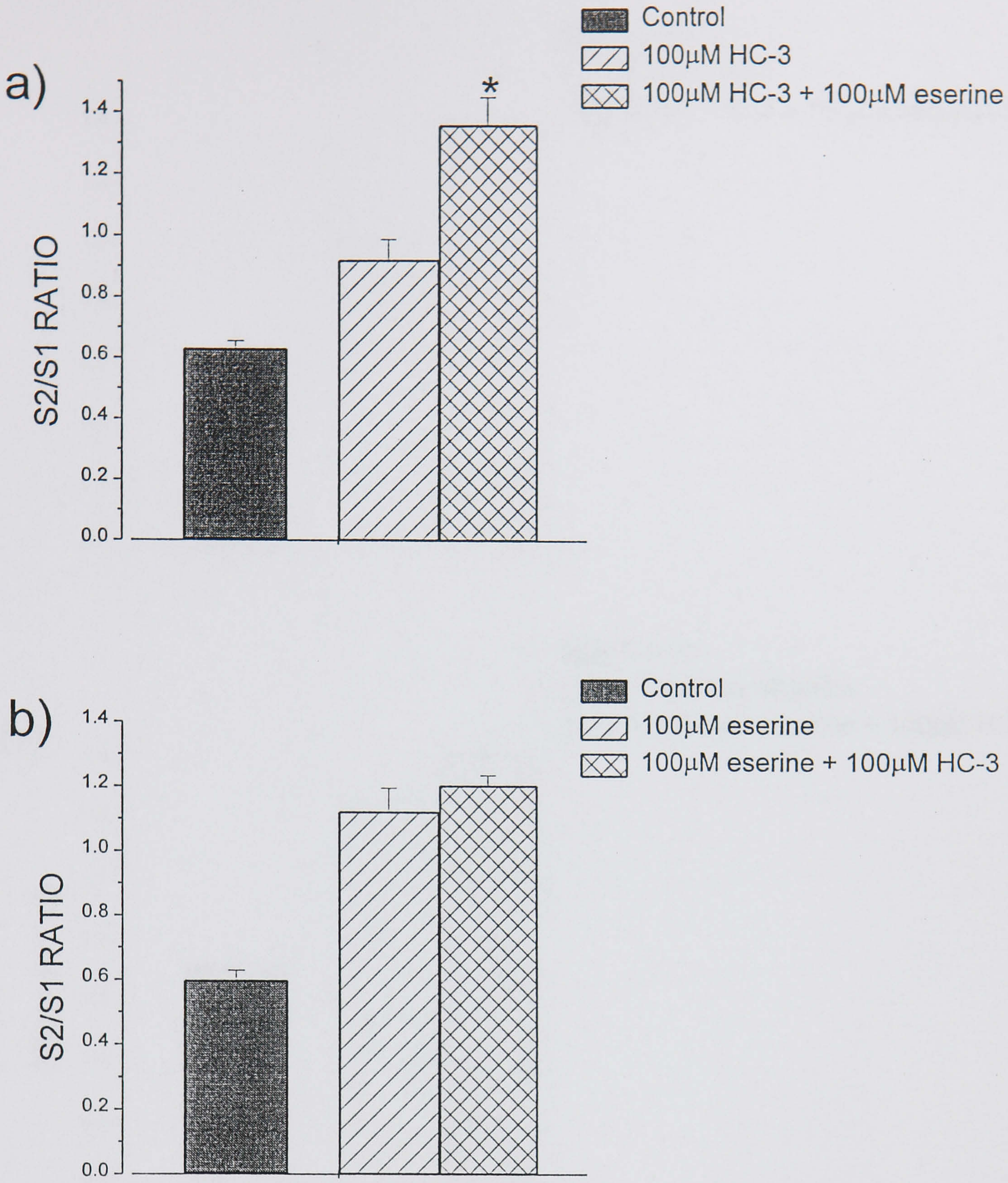


Figure 3.13 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effects of eserine and HC-3, individually and in combination.

a) HC-3 compared with HC-3 and eserine together

b) eserine compared with HC-3 and eserine together.

Each column represents the S2/S1 ratio as the mean ± SEM of means (N) obtained from 4 experiments; see Table 3.6 for details. Significant differences in the S2/S1 ratio compared to HC-3 or eserine alone are denoted by * $P < 0.05$.

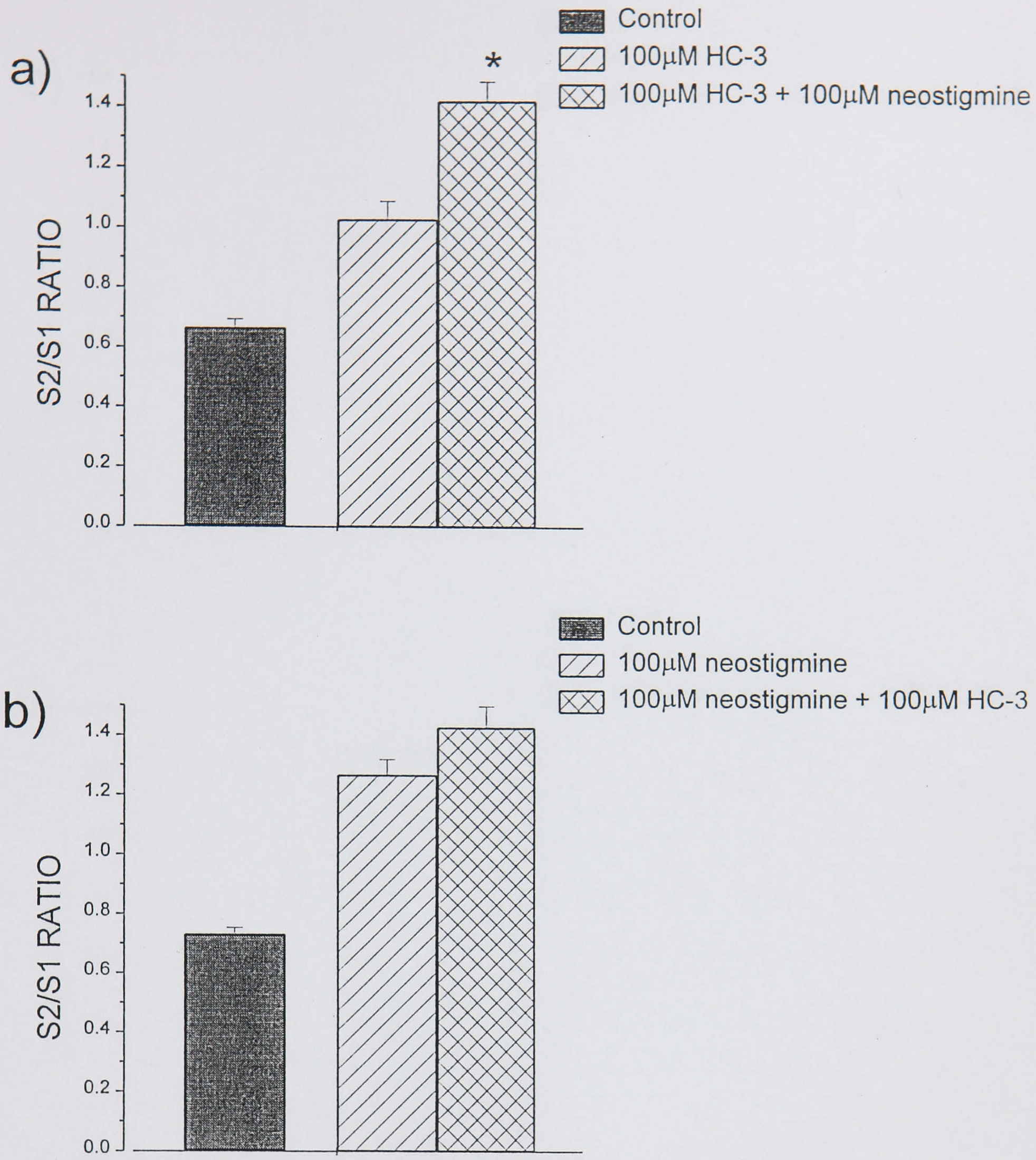


Figure 3.14 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effects of neostigmine and HC-3, individually and in combination.

a) HC-3 compared with HC-3 and neostigmine together

b) neostigmine compared with HC-3 and neostigmine together.

Each column represents the S2/S1 ratio as the mean ± SEM of the total number of replicate samples (n) obtained from 4 experiments; see Table 3.6 for details. Significant differences in the S2/S1 ratio compared to HC-3 or neostigmine alone are denoted by * $P < 0.001$.

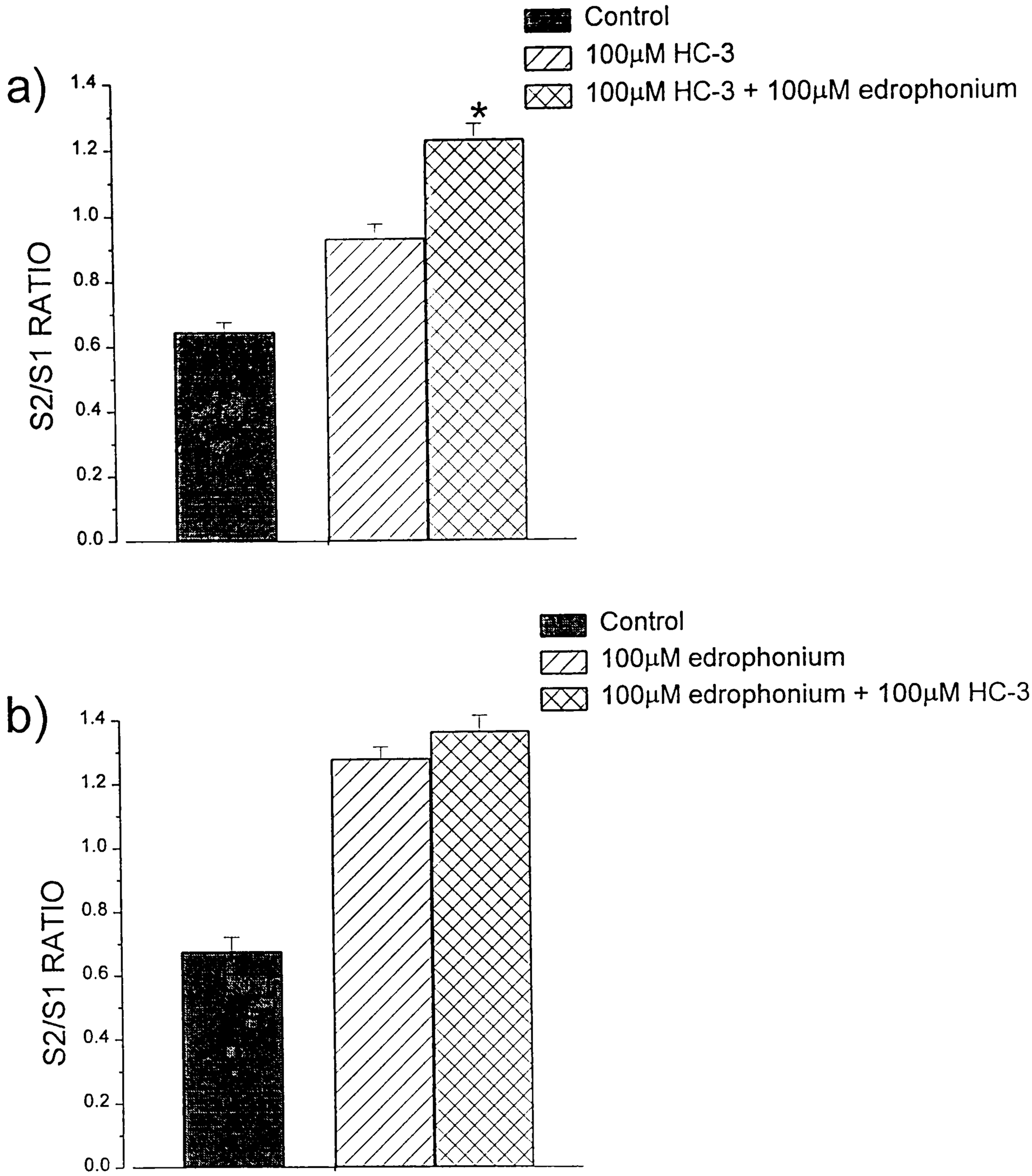


Figure 3.15 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effects of edrophonium and HC-3, individually and in combination.

a) HC-3 compared with HC-3 and edrophonium together

b) edrophonium compared with HC-3 and edrophonium together.

Each column represents the S2/S1 ratio as the mean ± SEM of the total number of replicate samples (n) obtained from 4 experiments; see Table 3.6 for details. Significant differences in the S2/S1 ratio compared to HC-3 or edrophonium alone are denoted by *P< 0.0001.

4. The effect of tacrine on [^{3}H]ACh release in reserpinized prisms.

As shown in Figure 3.16a, the comparison of the S2/S1 ratios in reserpinised and untreated prisms - reserpinisation of the prisms has no effect on the S2/S1 ratio of [^{3}H]ACh release (untreated S2/S1 0.706 ± 0.024 (n=18) and reserpinized S2/S1 0.661 ± 0.034 (n=17); P>0.05). Furthermore Figure 3.16b shows that 30μM tacrine and 100μM neostigmine potentiated [^{3}H]ACh release in reserpinized prisms in a similar manner to that in untreated prisms. Tacrine and neostigmine increased the S2/S1 ratios by 45 and 67% (control S2/S1 0.712 ± 0.040 (n=11), 30μM tacrine S2/S1 1.035 ± 0.100 (n=14), P<0.001 and 100μM neostigmine 1.191 ± 0.071 (n=15); P<0.001).

The concentration-effect curve for the effect of tacrine on [^{3}H]ACh release in reserpinised prisms is shown in Figure 3.17 and Table 3.7. In contrast to its effect in untreated prisms, 100μM tacrine had no significant effect on [^{3}H]ACh release in reserpinized prisms (P>0.05). An inhibition of 55% was observed with 200μM tacrine - reserpinization had no effect on the inhibition of [^{3}H]ACh release by tacrine at this concentration (P<0.0001), compare Figure 3.3.

CONCENTRATION TACRINE	S2/S1 RATIO	
	CONTROL	TACRINE
30μM	0.712 ± 0.040 (N=4)	1.035 ± 0.100* (N=4)
100μM	0.665 ± 0.013 (N=4)	0.614 ± 0.062 (N=4)
200μM	0.655 ± 0.013 (N=4)	0.301 ± 0.021** (N=4)

TABLE 3.7

The effect of tacrine on K^+-evoked [^{3}H]ACh release from reserpinized hippocampal prisms. The test samples were superfused with tacrine 10 min prior to and during the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of means (N) observed from four experiments; see Methods (Chapter 2) for details. Significant differences with respect to the controls are denoted by * P<0.001 and ** P< 0.0001.

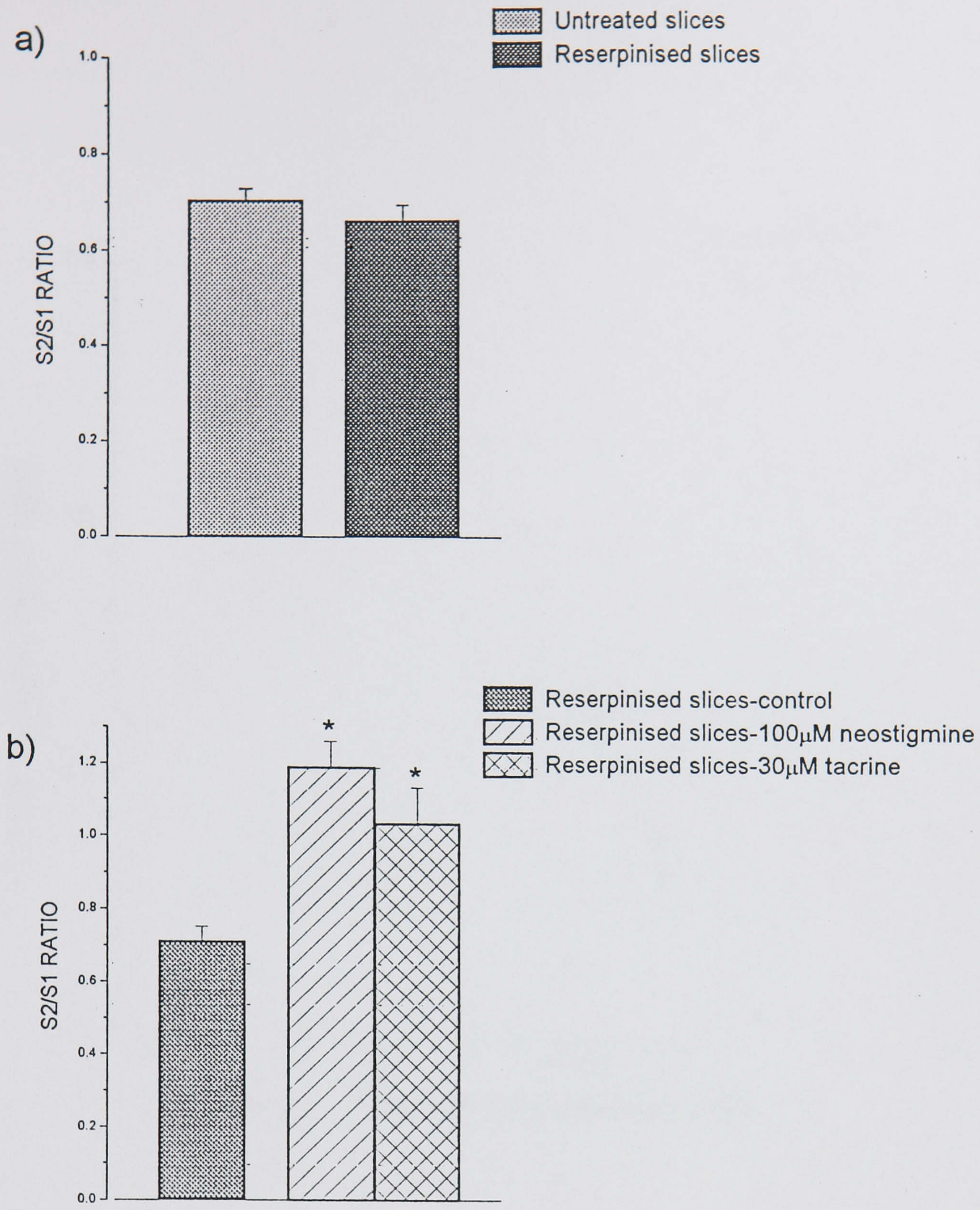


Figure 3.16 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of reserpinization.

a) reserpinized prisms (see Chapter 2 for experimental details) compared with untreated (non reserpinized) prisms

b) the effect of neostigmine and tacrine in reserpinized prisms.

Each column represents the S2/S1 ratio as mean ± SEM of the total number of replicate samples (n) obtained from 3 to 4 experiments; see Results for details. Significant differences in the S2/S1 ratio compared to the control are denoted by *P<0.001.

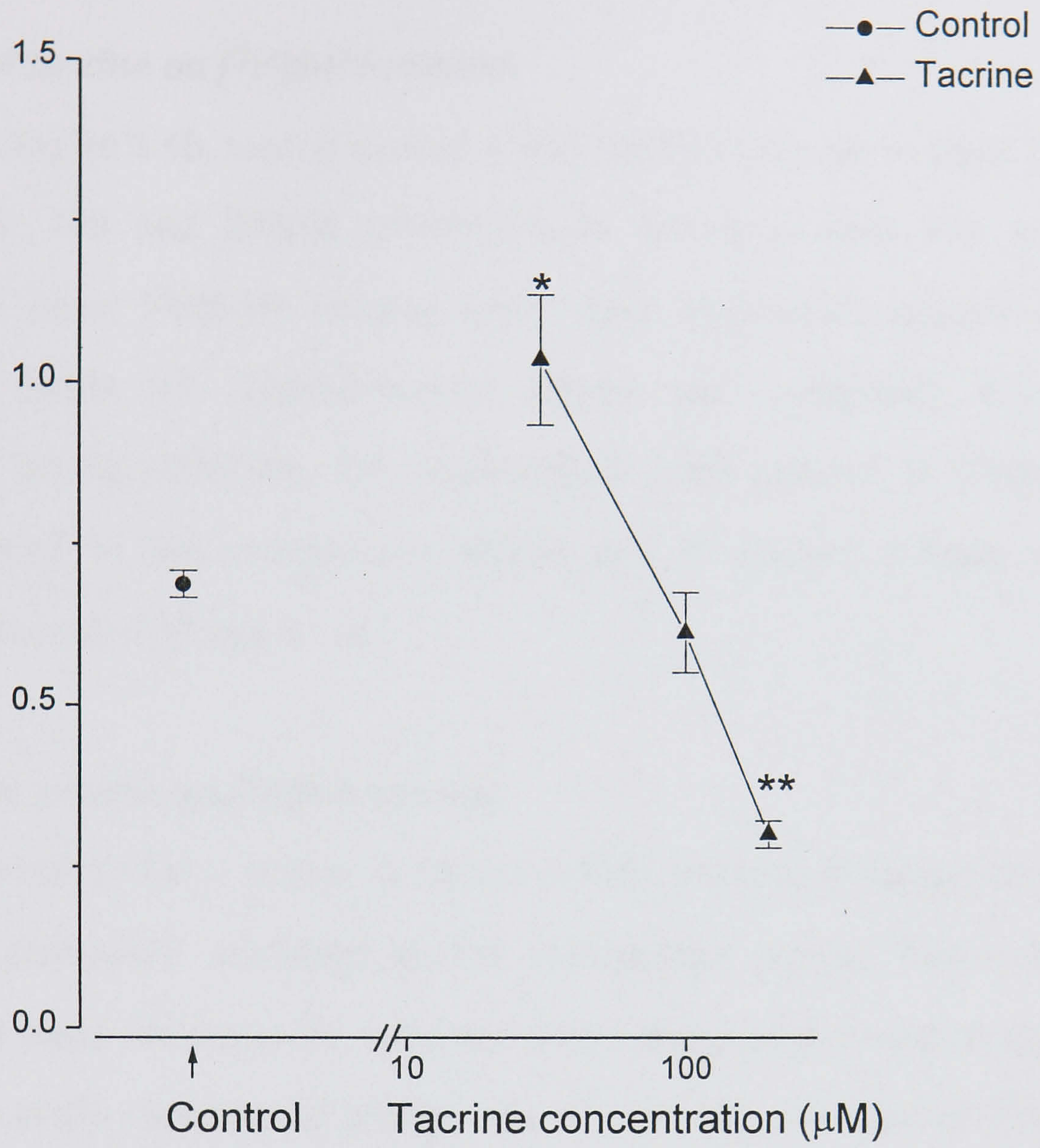


Figure 3.17 The S2/S1 ratios of K^+-evoked release of [^{3}H]ACh from reserpinized rat hippocampal prisms (see Chapter 2 for experimental details) - the effect of tacrine.

The prisms were stimulated twice (S1 and S2) for 2 min and tacrine was added to the superfusion medium 10 min prior to and during the S2 stimulation. Each point represents the S2/S1 ratio as the mean ± SEM of means (N) obtained from 4 experiments; see Table 3.7 for details. The control represents the mean ± SEM of means from all the experiments. Significant differences in the S2/S1 ratio compared to the control group are denoted by *P<0.001 and ** P<0.0001.

5. The effect of tacrine on monoamine and 5-HT release and the effect of reserpinising the hippocampal prisms.

The effect of tacrine on [^{3}H]5-HT release.

As shown in Figure 3.18, tacrine evoked a very marked increase in basal release of [^{3}H]5-HT. At 100 and 200μM concentrations tacrine caused 302 and 374% increases in basal [^{3}H]5-HT release which were significantly greater than that evoked by 30mM K^+. Tacrine-evoked release was completely abolished in reserpinized prisms. Similarly, the unstimulated basal release of [^{3}H]5-HT was greatly reduced in the reserpinized prisms and K^+-evoked release was also completely abolished (Figure 3.18).

The effect of tacrine on [^{3}H]NA release.

At 100μM concentrations, tacrine produced a 87% increase in basal release. This effect was completely abolished in the reserpinized prisms. Furthermore, the unstimulated basal release of the untreated (non reserpinized) prisms is significantly greater than in the reserpinized prisms. The K^+-stimulated release of [^{3}H]NA from the reserpinized prisms was completely abolished (Figure 3.19).

The effect of tacrine on [^{3}H]DA release

Tacrine at 100μM produced a 96% increase in the basal release of [^{3}H]DA, which was totally abolished in the reserpinized prisms. As with [^{3}H]NA, basal release from these prisms was markedly lower than that of the untreated prisms. The K^+-evoked release was also greatly reduced if not abolished from the reserpinized prisms (Figure 3.20).

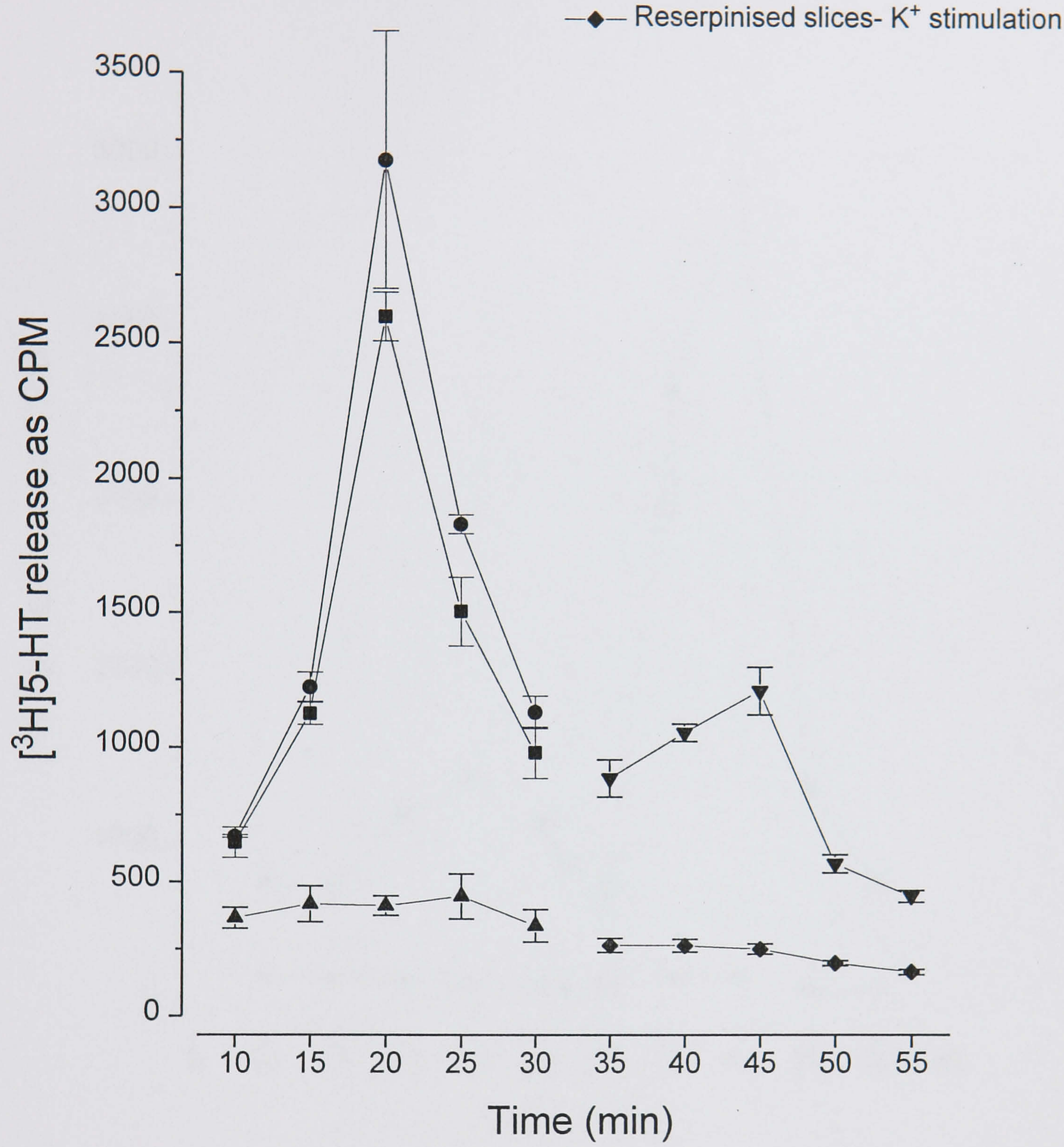


Figure 3.18 The effect of tacrine and 30mM K^+ on [^{3}H]5-HT release from reserpinized (see Chapter 2, Methods for experimental details) and untreated (non reserpinized) rat hippocampal prisms.
The prisms were stimulated twice (S1 and S2) for 2 min with tacrine (S1) and K^+ (S2). Each point represents release as the mean ± SEM of 6 replicate samples obtained from a typical experiment and expressed as CPM.

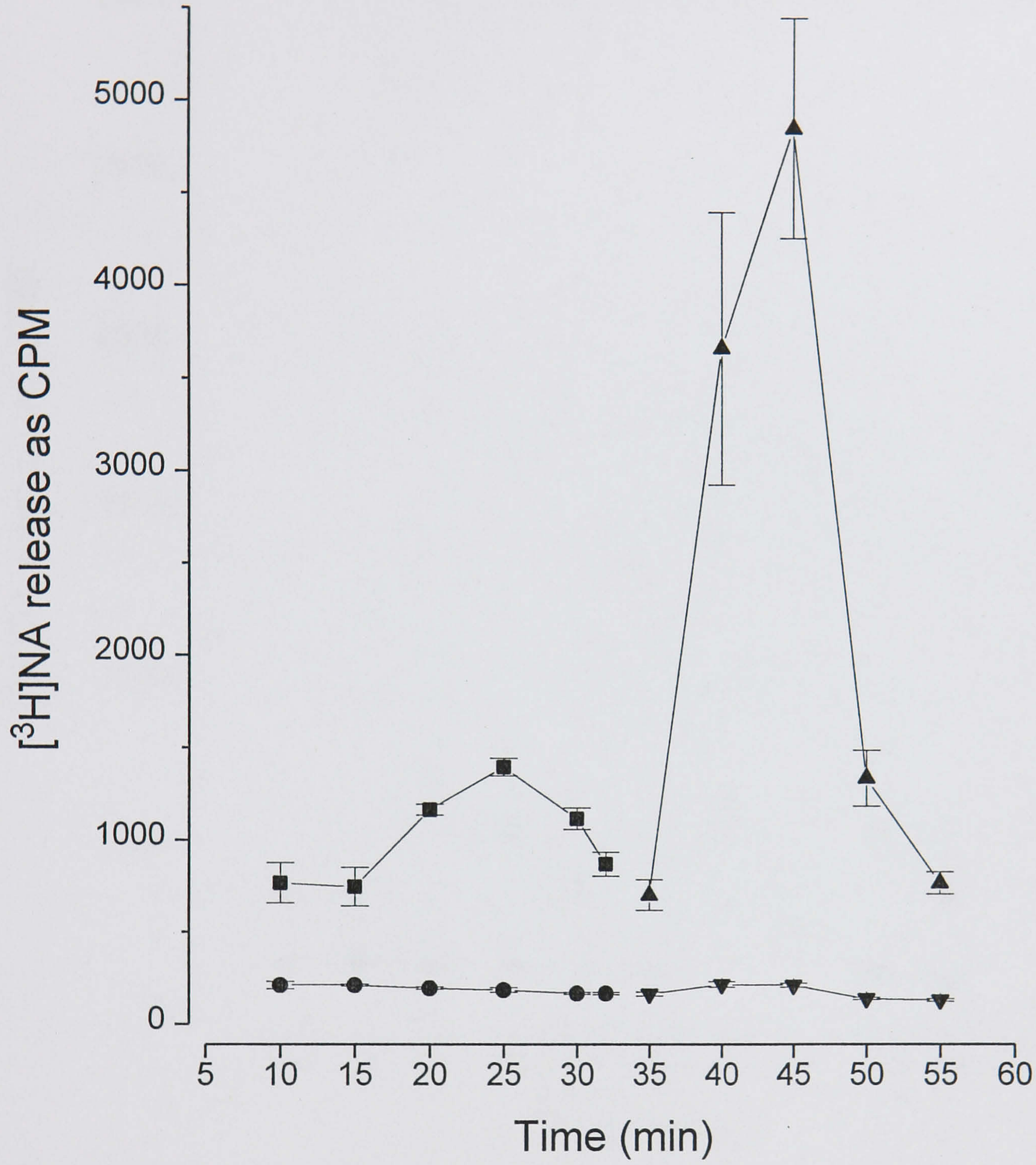


Figure 3.19 The effect of tacrine and 30mM K^+ on [^{3}H]NA release from reserpinized (see Chapter 2, Methods for experimental details) and untreated (non reserpinized) rat hippocampal prisms.
The prisms were stimulated twice (S1 and S2) for 2 min with tacrine (S1) and K^+ (S2). Each point represents release as the mean ± SEM of 6 replicate samples obtained from a typical experiment, expressed as CPM.

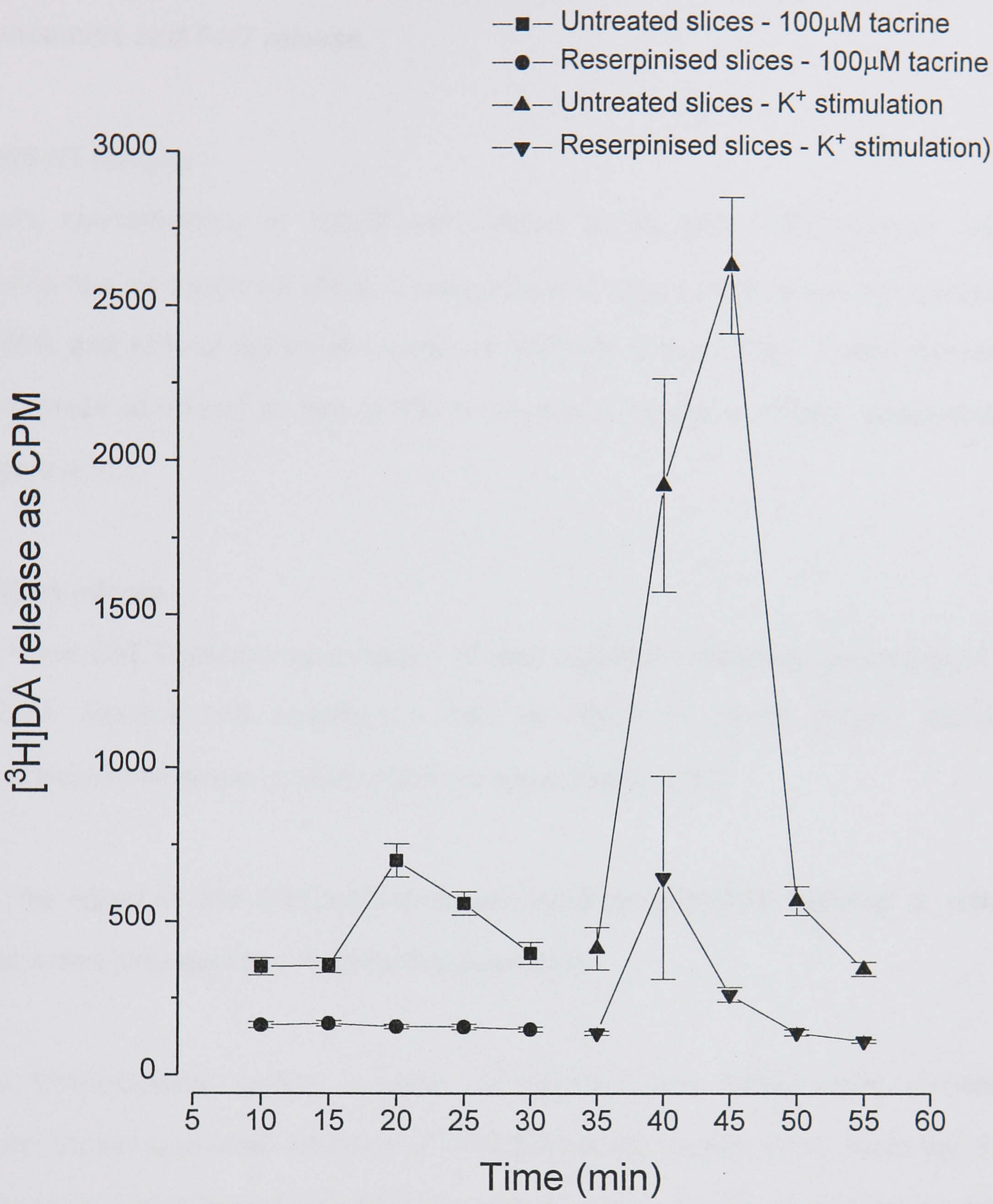


Figure 3.20 The effect of tacrine and 30mM K^+ on [^{3}H]DA release from reserpinized (see Chapter 2, Methods for experimental details) and untreated (non reserpinized) rat hippocampal prisms.
The prisms were stimulated twice (S1 and S2) for 2 min with tacrine (S1) and K^+ (S2). Each point represents release as the mean ± SEM of 6 replicate samples obtained from a typical experiment, expressed as CPM.

6. The effect of the ChE-inhibitors eserine, neostigmine and edrophonium on monoamine and 5-HT release.

[^{3}H]5-HT release

Again, concentrations of 100μM were tested for all three ChE-inhibitors. While eserine had no significant effect, neostigmine and edrophonium produced increases of 58% and 45% of the basal release of [^{3}H]5-HT (Figure 3.21). These increases were small compared to that of 302%, seen with tacrine at similar concentration (Figure 3.18).

[^{3}H]NA release.

All three ChE-inhibitors were tested at their maximum effective concentration of 100μM. Eserine and neostigmine had no effect on basal [^{3}H]NA release. Edrophonium however, caused a 39% increase (Figure 3.22).

7. The effect of the ChE-inhibitors and HC-3 on [^{3}H]QNB binding in whole membrane preparations from rat hippocampus.

The ChE-inhibitors tacrine, e serine, neostigmine and edrophonium showed a concentration-dependent inhibition of [^{3}H]QNB binding (Figure 3.23). From the data presented tacrine appears to have the highest affinity for the muscarinic receptor binding sites. The affinity of HC-3 to these binding sites is comparable to the ChE-inhibitors eserine, neostigmine and edrophonium. Atropine was used to define specific binding and saturation was observed at 1μM atropine (data not shown).

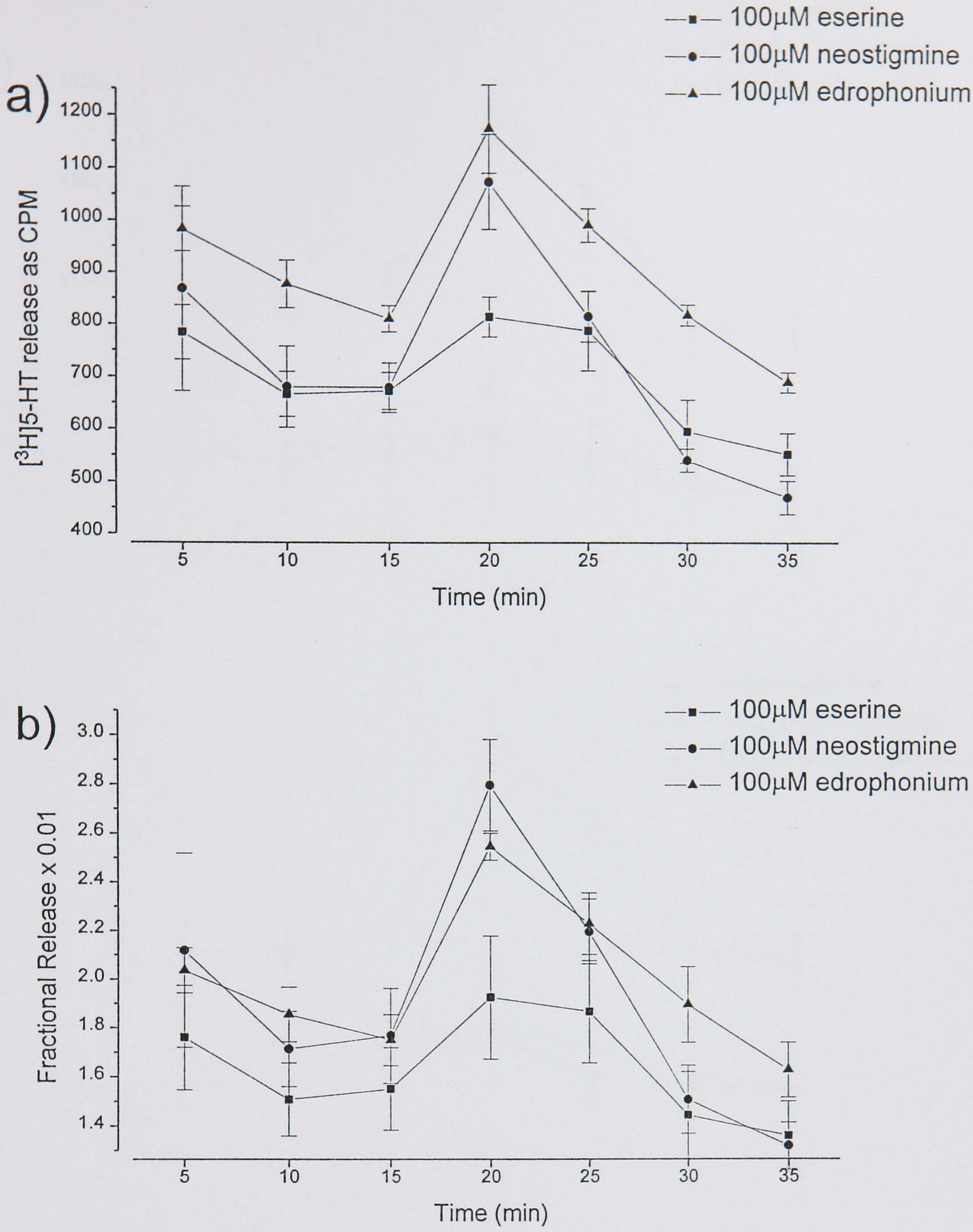
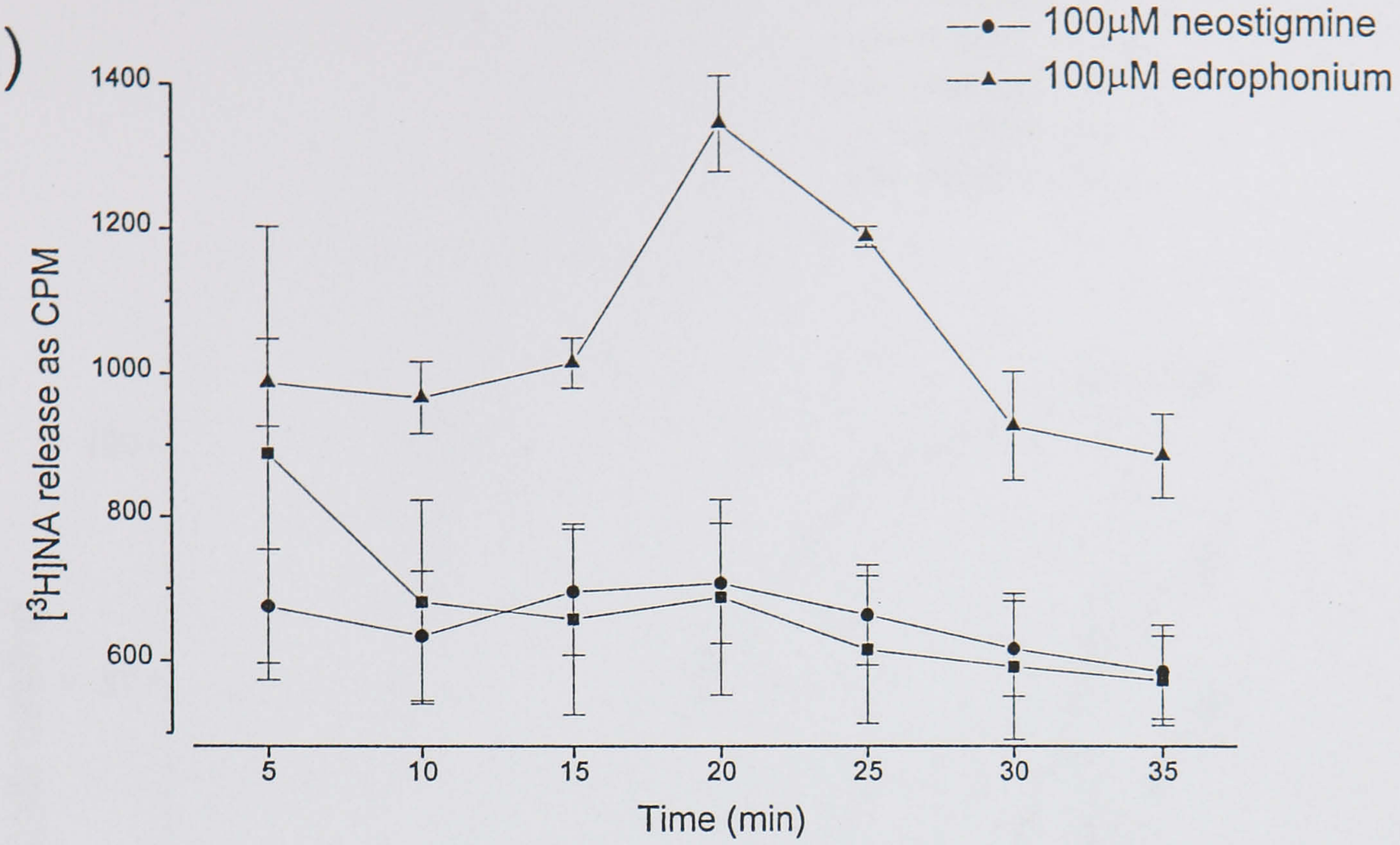


Figure 3.21 The effect of the ChE-inhibitors eserine, neostigmine and edrophonium on [^{3}H]5-HT release from rat hippocampal prisms.

The tissue prisms were superfused with each of the ChE-inihibtors for 2 min. Each point represents release as the mean ± SEM of replicate samples obtained from a typical experiment, represented as:

a) CPM

b) fractional release.



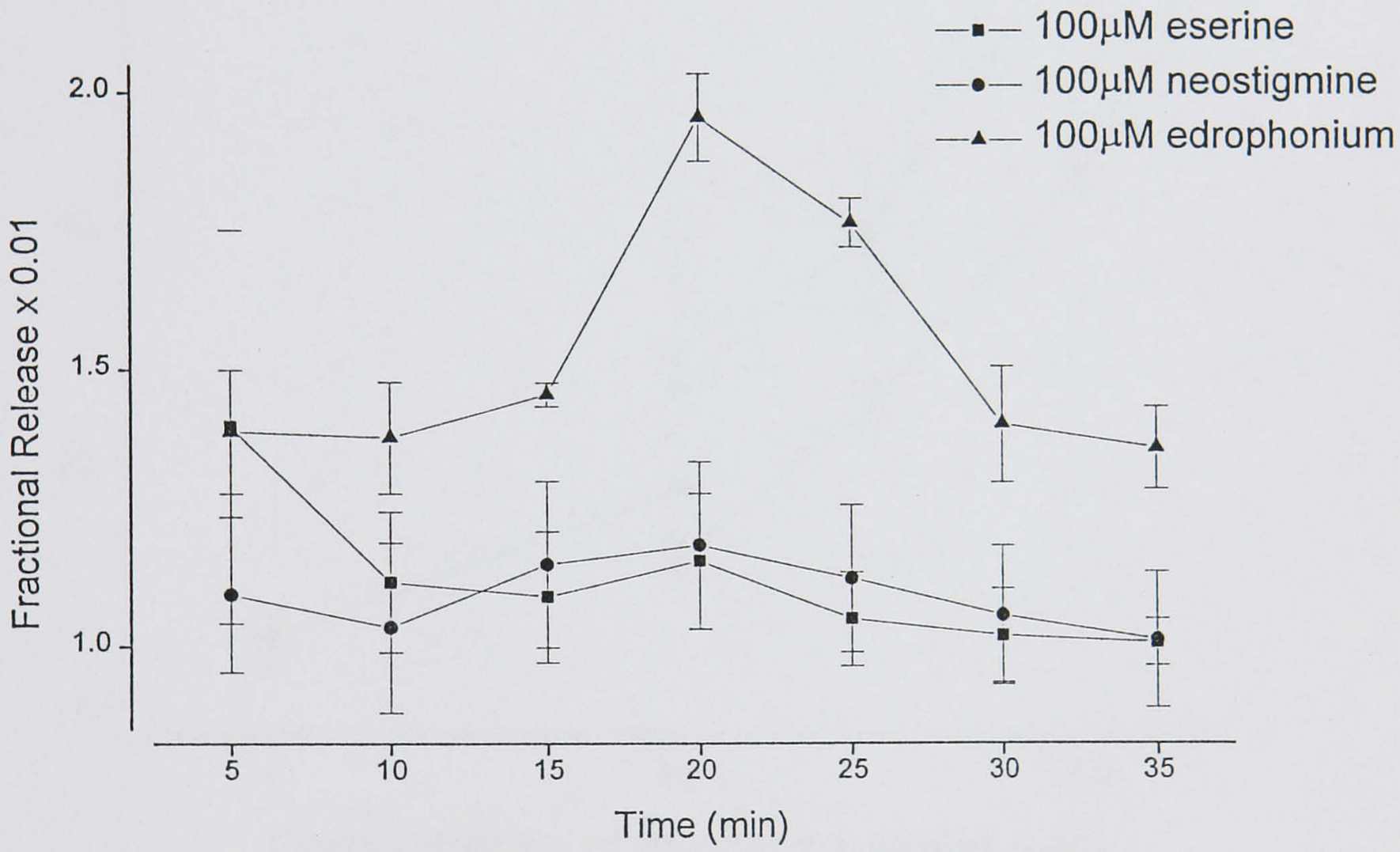


Figure 3.22 The effect of the ChE-inhibitors eserine, neostigmine and edrophonium on [^{3}H]NA release from rat hippocampal prisms.

The tissue prisms were superfused with each of the ChE-inihibtors for 2 min. Each point represents release as the mean ± SEM of replicate samples obtained from a typical experiment, represented as:

a) CPM

b) fractional release.

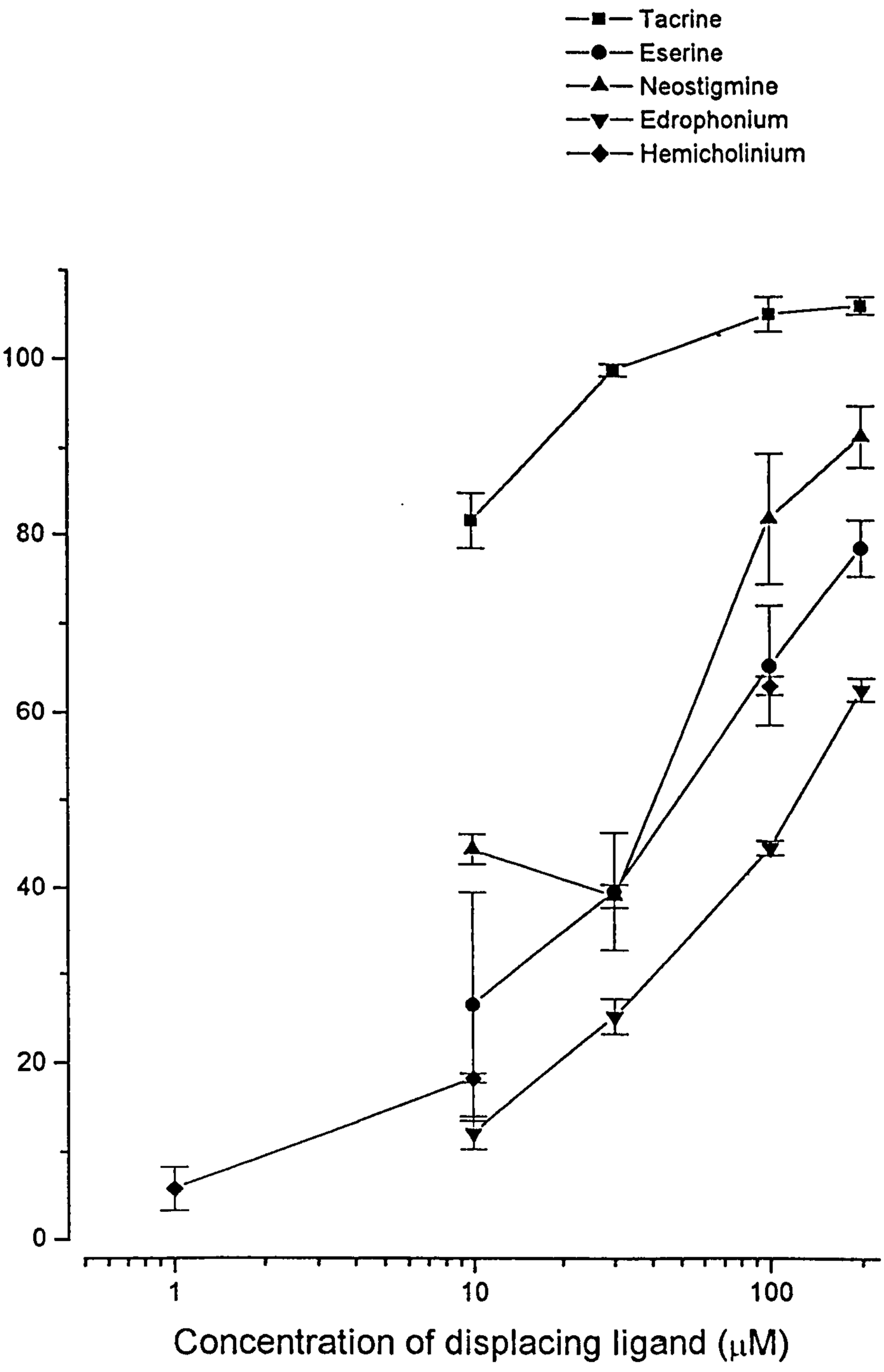


Figure 3.23 Displacement of [^{3}H]QNB binding in rat hippocampal membrane preparations by ChE-inhibitors and HC-3.

Each point represents % inhibition as the mean ± SEM obtained from 3 experiments, each done in quadruplicate.

DISCUSSION

The potentiation of [^{3}H]ACh release by the ChE inhibitors

The results presented in this chapter represent the first systematic comparison of the concentration-effect relationship for structurally different ChE-inhibitors acting to potentiate the K^+-evoked release of ACh in rat hippocampal prisms. The ChE-inhibitors tacrine, eserine, neostigmine and edrophonium all produce clear increases in K^+-evoked [^{3}H]ACh release from hippocampal prisms (Figures 3.1 - 3.10) despite previous conflicting reports (see Introduction to this chapter).

These increases were concentration dependent with neostigmine and edrophonium in the range 10-200μM. Eserine however, gave no further increase in effect at concentrations above 10μM. Taken together, the S2/S1 ratios obtained (Figures 3.3 - 3.15) show no clear distinction in potency between the ChE-inhibitors in promoting [^{3}H]ACh release. Their potencies as enzyme inhibitors however have been reported to differ widely. Thus the IC_{50} of neostigmine in 30 min incubations with human brain ChE, is smaller, by one and two orders of magnitude than that of tacrine and edrophonium respectively. The IC_{50}s for tacrine, eserine, neostigmine and edrophonium are 0.24μM, 72nM, 35nm and 3.7μM respectively. (Atack *et al.,* 1989). It would appear therefore, that the concentrations of the ChE-inhibitors and reaction times employed in the present release experiments, are sufficient to cause full inhibition of ChE during the 12-min exposure to the tissue. More interestingly however these release experiments show no clear difference between the effects of the two ChE-inhibitors likely to be restricted to extracellular actions and the two likely to have additional intracellular actions.

In comparison with the other three ChE-inhibitors, tacrine at concentrations above 50μM showed clear inhibition of [^{3}H]ACh release, as shown in Table 3.1 and Figures

3.2 & 3.3. Interestingly, bell-shaped dose-response curves for the memory-enhancing effects of tacrine have also been observed in animal studies (Nielsen *et al.*, 1989). The inhibition of release observed with tacrine is in agreement with many previous observations where tacrine inhibited the electrically stimulated release of [^{3}H]ACh in cortical (Drukarch *et al.,* 1987; Loiacono & Mitchelson, 1990), striatal (Drukarch *et al.,* 1987; Buyukuysal & Wurtman, 1989) and hippocampal (Buyukuysal & Wurtman, 1989; Potter *et al.,* 1989) tissue. It has been proposed that ChE-inhibitors such as eserine and tacrine might inhibit the release of [^{3}H]ACh from brain slices by activating presynaptic autoreceptors in response to increased concentrations of ACh in the synaptic cleft (Nilsson *et al.,* 1987; Szerb & Somogyi, 1973). However all four ChE-inhibitors clearly increased [^{3}H]ACh release in the present experiments and to approximately the same extent. It is difficult therefore to see how increased concentrations of extracellular ACh, caused by tacrine could activate autoreceptors sufficiently to cause overt inhibition of release, while similar increases in extracellular ACh caused by the other inhibitors did not cause similar inhibition. The present results therefore make it clear that tacrine exerts some additional effect not caused by the other ChE-inhibitors.

The increase in K^+-evoked release caused by these ChE inhibitors may be thought to be due to the accumulation of [^{3}H]ACh in the synaptic cleft as a result of the inhibition of its hydrolysis, since extracellular [^{3}H]ACh is not transported by choline carriers (Simon *et al.,* 1975). It would therefore be available for collection in the tissue superfusates, unlike the [^{3}H]choline formed as a result of its hydrolysis, in the absence of ChE inhibitors. An appreciable percentage of [^{3}H]choline formed in this way in the synapse would be expected to be less susceptible to collection because it would be transported into the intact cells in the tissue prisms. Bearing this in mind, it was considered that hemicholinium-3 (HC-3) might have a comparable or even greater effect on the S2/S1 ratio than did the ChE inhibitors.

As expected HC-3 alone at concentrations of 10-100μM gave significant increases in the S2/S1 ratio, although perhaps surprisingly these increases were not as marked as those caused by the ChE inhibitors alone (Figure 3.11). It is likely that this increase in S2/S1 ratio reflects the [^{3}H]choline accumulated, as a result of the hydrolysis of the released [^{3}H]ACh, and the inhibition of its transport, since it has been shown that [^{3}H]choline efflux does not increase during stimulation (see Chapter 2, General Methods and Materials). Interestingly the inclusion of a ChE-inhibitor with HC-3 gave a further significant increase over and above that caused by HC-3 alone (Figures 3.12a-3.15a). This effect was consistently observed with all four ChE-inhibitors. In contrast, each combination of HC-3 and ChE inhibitor gave no greater increase in S2/S1 ratio than was obtained with the corresponding ChE inhibitors acting alone (Figures 3.12b-3.15b). These results indicate that the observed effect of the ChE-inhibitors is not entirely due to the inhibition of cholinesterase and the resulting accumulation of [^{3}H]ACh in the tissue extracellular space. It would appear therefore that the ChE-inhibitors have an additio nal direct effect in promoting [^{3}H]ACh release.

A previous study by Umeda and Sumi (1990), similarly examined the effects of HC-3 and eserine on basal and K^+-stimulated release of unlabelled ACh from striatal and hippocampal slices. Release was evoked into a fixed volume of K^+-enriched incubation medium, rather than into medium superfusing slices. It was shown that HC-3 (100μM) and eserine (20μM) independently increased the basal and K^+-stimulated release of ACh although together, they caused an additive effect on the stimulated release of ACh. This is partly similar to the results reported here with the superfusion method, where the effect of HC-3 (100μM) and eserine (100μM) in combination was clearly and significantly greater than HC-3 (100μM) alone but not greater than 100μM eserine. A point of difference is that the present experiments show no effect of HC-3 or ChE-inhibitor on basal release.

HC-3 is a well known, potent inhibitor of high affinity choline uptake into nerve terminals and has been shown to decrease ACh release gradually by attenuating synthesis, which depends predominantly, if not entirely on the choline uptake from extracellular fluid (Kuhar & Murrin, 1978; Tedford *et al.,* 1986). The present results show no evidence that the brief incubations (12 min) with HC-3 reduce the availability of [^{3}H]ACh for release. It is important to remember that the prisms are preloaded with [^{3}H]choline some 2h before release is initiated. HC-3 added after these 2h will be unable therefore, to cause a significant reduction in the quantity of [^{3}H]ACh available for release.

To the contrary, it can be argued that the HC-3 eventually added to the superfusing medium, will reduce ongoing ACh synthesis from largely unlabelled choline. In the absence of HC-3, choline will continue to be released and reuptaken into the prisms after excess [^{3}H]choline has been removed by washing the tissue (Chapter 2). HC-3 could be seen therefore, as increasing the availability of [^{3}H]ACh for release by reducing its gradual dilution with unlabelled ACh. It could be that prevention of 'radiolabel-dilution' contributes to the potentiation by HC-3 of the [^{3}H]ACh release actually observed, and more conventionally ascribed to block of the reuptake of [^{3}H]choline. However the contribution of this second mechanism is likely to be small, since in the present experiments HC-3 is only in contact with the tissue for 12 min, after 84 min (see Chapter 2) of release and uptake of predominantly unlabelled choline.

HC-3 is also known to have muscarinic receptor blocking properties (Bieger *et al.,* 1968; Bertolini *et al.,* 1967; Madden & Mitchelson, 1975). It is possible therefore that it promotes ACh release by direct block of presynaptic muscarinic autoreceptors, which are believed to cause feedback inhibition of ACh release upon activation. However this seems unlikely since atropine (10μM), which alone has no effect on K^+-evoked [^{3}H]ACh release, is demonstrated in Chapter 5 to increase further, the

release of [^{3}H]ACh in the presence of ChE-inhibitors. As discussed previously HC-3 did not increase the S2/S1 ratio in the presence of any of the ChE-inhibitors tested (Figures 3.12b - 3.15b).

ChE-inhibitors (e.g eserine and soman) acting in peripheral synapses such as those in the skeletal muscle for a prolonged period of time (usually over 1 h), have been shown not only to preserve the release of ACh but also to cause a gradual rise of the ACh content in the tissue (Molenaar *et al.,* 1987) in resting or stimulated conditions, by penetrating the nerve terminal. The extra amount of ACh accumulating after ChE inhibition is referred to as 'surplus ACh', to be differentiated from the 'depot ACh' (ACh store) normally involved in synaptic transmission. Molenaar *et al.,* (1987), concluded from their studies that the surplus ACh is synthesized and stored in the motor nerve endings and is the main source of the resting (basal) release of ACh. In agreement with previous observations they were also able to demonstrate that surplus ACh was not released by nervous impulses but was partially released by high concentrations of KCl applied for long periods of time (50mM, for 60 min), in a Ca^{2+} dependent way. Similar findings have also been shown by Collier and Katz (1971) for the superior cervical ganglion although in the brain, surplus ACh has not been demonstrated (Bourdois & Szerb, 1972). The possibility that 'surplus ACh' may contribute to the increased K^+-evoked [^{3}H]ACh release following the action of the cholinesterases, was however still considered.

A number of observations argue against the above interpretation. First, there was no evidence of an increase in basal or resting release following the addition of the ChE-inhibitors 10 min before the S2 stimulation. Second, the ChE-inhibitors neostigmine and edrophonium also increased [^{3}H]ACh release. These ChE-inhibitors being quaternary ammonium compounds are permanent cations and are likely to have limited access if any, into nerve terminals especially during short periods of superfusion. They would therefore be expected to act predominantly extracellularly,

rather than on the intracellular stores. Moreover the time period for such an accumulation of 'surplus ACh' might be expected to be greater than 12 min, as previous reports suggest. It was also observed (Figure 3.8b) that superfusion of the slices with neostigmine for only 2 min during the S2 stimulation, still increased the release of [^{3}H]ACh. It appears unlikely therefore, that surplus ACh contributes to the increase in release caused by the ChE-inhibitors reported in this chapter.

Another possibility which may contribute to the increase observed in the S2/S1 release ratio is an increase in [^{3}H]choline efflux either by a direct action of the ChE inhibitor itself or indirectly, by elevated synaptic [^{3}H]ACh concentrations activating presynaptic muscarinic receptors. Ladinsky *et al.* (1974) demonstrated that oxotremorine and eserine increased tissue choline levels not only in ACh rich areas in mouse brain, but also in tissues such as spleen and cerebellum which contain no ACh, or very low levels. They concluded that feedback inhibition was not a necessary requirement for raising choline levels, since DFP (dyflos) was without effect. It was also argued that decreased turnover of ACh (retarded incorporation of choline into ACh following inhibition of release) was unlikely to be the cause as oxotremorine increased choline levels even in the spleen which contains no ACh as far as is known (Ladinsky *et al.,* 1974). Similar observations have been made using rat striatal slices, treated with high concentrations of oxotremorine (1, 5mM) or ACh (1mM, after treatment with ChE inhibitors) for prolonged incubation times (20 min) (Dolezal & Tucek, 1984). It was concluded by these investigators that stimulation of muscarinic receptors increased extracellular choline concentrations by an effect on intracellular phospholipid metabolism.

In contrast to the above, studies by Weiler (1989) on the release of endogenous ACh from striatal slices pretreated with ChE inhibitors have demonstrated that choline release during ChE inhibition was unchanged during K^+ depolarisation when compared to that released spontaneously. Furthermore, it was shown that choline

efflux actually decreased in the presence of the muscarinic agonist oxotremorine-M at concentrations in the micromolar range. The discrepancy between Weiler's results and those described above has been attributed to the experimental conditions (Weiler, 1989). The stimulation time was longer where increased choline efflux was observed, (Brown *et al.,* 1988; Lindmar *et al.,* 1986; Dolezal & Tucek, 1984) and the concentrations of the muscarinic agonists were also much larger than those used in Weiler's study. The conditions used in this present study and described in Chapter 2 are more comparable to Weiler's, where the exposure to high K^+ was only for a period of 5 min.

Furthermore, the data presented in this chapter are not compatible with an increase in [^{3}H]choline efflux contributing to the increase of K^+-evoked release observed in the presence of the ChE inhibitors. [^{3}H]Choline released would be susceptible to reuptake so that full potentiation by ChE inhibitors would only be evident in the presence of HC-3. The results in fact show (Figures 3.12b - 3.15b), that HC-3 in the presence of the ChE inhibitors did not increase the potentiation of release any further. It is therefore concluded that the increase in the S2/S1 release ratios, observed in the presence of the ChE inhibitors, is unlikely to be due to an increase in [^{3}H]choline efflux.

It would appear therefore that under the present experimental conditions, the ChE inhibitors may have some unknown activity stimulating ACh release. Previous reports suggesting that eserine and neostigmine have actions other than those of inhibition of ChE enzymes, seem to support this notion. Bell (1966), found that eserine (10 and 100μg/ml) produced an increase in tone of the innervated toad bladder, by releasing acetylcholine from cholinergic nerve endings. A similar mechanism of action for the eserine-induced spasm of guinea-pig trachealis muscle was proposed by Carlyle (1963). Neostigmine has been shown to stimulate sympathetic ganglia (Mason, 1962a & b) and also to act in a similar way to eserine on the guinea-pig trachealis

muscle (Carlyle, 1963). Cox and Lomas (1972), investigated more fully the pharmacological actions of these two drugs, by studying the concentration-effect relations on the guinea-pig isolated ileum. It was concluded that the eserine and neostigmine-induced spasms of the ileum smooth muscle were concentration-related and due to an indirect action involving the release of ACh from cholinergic nerves. However the inhibition of ChE appeared not to be important for this action as experiments with dyflos, at a concentration that completely inhibited the ability of ileum homogenate to hydrolyse ACh, did not modify either the eserine or neostigmine dose-response plots. It was thus concluded, like this present chapter, that the higher levels of ACh obtained in release experiments might be related to a mechanism other than the efficient inhibition of ChE. It is interesting to note that this conclusion, separately arrived at, has practical implications. It must be borne in mind that ACh release measured in the presence of ChE-inhibitors differs from physiological release by the superimposition of at least two pharmacological mechanisms and would therefore be best treated with extreme caution.

The inhibition of [^{3}H]ACh release by high concentrations of tacrine

As mentioned previously in this discussion, tacrine at concentrations above 50μM showed an inhibition of [^{3}H]ACh release - represented in its bell-shaped concentration-effect curve (Figure 3.3), which is quite unlike those of the other ChE-inhibitors (Figures 3.4 - 3.10). At a concentration of 100μM, tacrine caused a 21% reduction of the S2/S1 ratio below control values ($P<0.001$). At 200μM tacrine, the reduction was far greater, 66% below control values ($P<0.0001$). As concluded earlier in this discussion, inhibition of this magnitude cannot be ascribed to feedback inhibition caused by elevated concentrations of ACh even though previous authors have implicated muscarinic cholinoceptors in this role. However a direct action of tacrine itself on such receptors should be considered.

To cause such inhibition, tacrine would have to act presynaptically as a muscarinic agonist. The [^{3}H]QNB-displacement curves (Figure 3.23) show tacrine to have appreciably stronger muscarinic receptor binding properties than the other ChE-inhibitors. However tacrine has been shown to inhibit the carbachol-stimulated hydrolysis of phosphatidylinositol (PI) in cortical slices (Freeman & Dawson, 1991). This suggests tacrine to be a muscarinic receptor antagonist rather than an agonist, even though there is evidence that PI hydrolysis is stimulated via muscarinic receptors other than those controlling ACh release (Hulme *et al.,* 1990). It is possible that muscarinic receptor blocking properties of tacrine and such compounds may play a role in potentiating ACh release. Muscarinic actions of tacrine are discussed further in Chapter 5. However alternative mechanisms by which tacrine might inhibit ACh release are considered below and in Chapter 4.

The interaction of tacrine with monoamines and 5-HT

In addition to its inhibitory effect on AChE and its interaction with muscarinic cholinoceptors, tacrine has also been shown to influence monoamine (Drukarch *et al.,* 1988) and GABA release (De Belleroche & Gardiner, 1988). Drukarch *et al.*, (1988), have shown tacrine to release [^{3}H]NA and [^{3}H]5-HT from cortical slices and [^{3}H]DA from striatal slices and similar observations have been made by Robinson *et al.,* (1989). The results in this present chapter show that tacrine at 100μM and 200μM concentrations, is also capable of producing direct release of [^{3}H]DA, [^{3}H]5-HT and [^{3}H]NA from hippocampal prisms in the absence of depolarization by K^+ (Figures 3.18 - 3.20). Furthermore the effect of tacrine on basal [^{3}H]5-HT release was significantly greater than the release evoked by 30mM K^+ (Figures 3.18). In comparison, while edrophonium showed comparable [^{3}H]NA releasing properties, neostigmine and eserine lacked effect on basal release (Figure 3.22). It was also observed that neostigmine and edrophonium caused release of [^{3}H]5HT, while eserine had no effect (Figure 3.21). For these reasons, a number of release

experiments included the pretreatment of the hippocampal prisms with 300nM reserpine, in order to reduce as far as possible, the influence of monoamines on ACh release. It was shown that reserpinization of the prisms abolished the effect of tacrine on basal release and also greatly diminished the K^+-evoked release of [^{3}H]5-HT, [^{3}H]NA and [^{3}H]DA (Figures 3.18 - 3.20).

A number of *in vitro* and *in vivo* studies have shown ACh release to be modulated by nerve terminal heteroreceptors for 5-HT, DA and NA. While most *in vivo* studies using microdialysis consistently demonstrate that the release of hippocampal ACh is enhanced by DA and 5-HT (Ohue *et al.,* 1992a & b), many *in vitro* studies have shown that 5-HT receptors mediate inhibition of ACh release in cortical and hippocampal tissue from rat and human brain (Maura & Raiteri, 1986; Barnes *et al.,* 1989; Maura *et al.,* 1992). In the rat hippocampus, 5-HT receptors of the 5-HT$_{1B}$ subtype have been shown to inhibit the depolarization-evoked release of [^{3}H]ACh (Maura & Raiteri, 1986). It is reported that tacrine however, does not display any affinity for adrenergic, dopaminergic or serotonergic receptors at concentrations which release [^{3}H]NA, [^{3}H]DA or [^{3}H]5-HT (Drukarch *et al.,* 1988).

Reserpinization of the prisms prior to release of [^{3}H]ACh however, had no effect on either the S2/S1 ratios for control release (Figure 3.16a), or the tacrine-induced potentiation (30μM) and inhibition (200μM) (Figure 3.17) of [^{3}H]ACh release. The concentration-effect curve for tacrine (Figure 3.17) is virtually identical to that in non-reserpinized slices (Figure 3.3). Potentiation by neostigmine remained at levels comparable with those in non-reserpinized slices (Figure 3.16b). It is therefore concluded that inhibition of [^{3}H]ACh release by tacrine, is unlikely to be due to an interaction with the monoamines NA, DA and 5-HT. Similarly it is unlikely that potentiation of [^{3}H]ACh release by ChE-inhibitors is due to an interaction with 5-HT, NA or DA, since neostigmine (100μM) and tacrine at 30μM were still able to increase the release of [^{3}H]ACh in reserpinized slices as shown in Figure 3.16b. Moreover

eserine, which does not affect the basal release of [^{3}H]5-HT or [^{3}H]NA (Figures 3.21 & 3.22) like tacrine or edrophonium, is just as effective at potentiating [^{3}H]ACh release in untreated slices (Figures 3.1 - 3.10).

The results presented in this chapter quite clearly show that the ChE inhibitors tacrine, eserine, neostigmine and edrophonium potentiate the release of K^+-evoked [^{3}H]ACh from hippocampal prisms. Furthermore it was also shown that tacrine unlike the other ChE-inhibitors, caused inhibition of [^{3}H]ACh release at high concentrations. The mechanism of action which causes the potentiation and inhibition of K^+-evoked [^{3}H]ACh release is however unknown as yet and remains to be elucidated. As discussed above, the inhibition and potentiation of [^{3}H]ACh release was not due to the release of monoamines or 5-HT by tacrine and the other ChE-inhibitors. It was also argued that the inhibition by tacrine was not due to muscarinic autoreceptor-mediated feedback inhibition by [^{3}H]ACh. However tacrine has also been shown to interact with a number of other receptor systems such as muscarinic, nicotinic, phencyclidine (PCP), adenosine and histamine receptors (Nordberg *et al.,* 1989). The aim of the following chapters therefore, was to compare the effects, on ACh release, of compounds known to have PCP, muscarinic or nicotinic actions with those of tacrine and the other ChE-inhibitors.

CHAPTER 4

The effect of σ ligands on [^{3}H]ACh release and its potentiation by ChE-inhibitors

INTRODUCTION

In the experiments described in Chapter 3 it was shown that the ChE-inhibitors tacrine, eserine, neostigmine and edrophonium all potentiate the K^+-evoked release of [^{3}H]ACh from hippocampal prisms. However it was also noted that at higher concentrations, tacrine, unlike the other ChE-inhibitors, caused a marked inhibition of release. As discussed in Chapter 3, tacrine has been shown to interact with muscarinic receptors as well as with a variety of other receptors. Previous studies investigating tacrine and the other ChE-inhibitors have suggested that they interact with the phencyclidine (PCP) site linked to the NMDA (N-methyl-*D*-aspartate) receptor channel complex. The aim of the work described in this chapter was to investigate the effects of PCP and PCP-like compounds on [^{3}H]ACh release and their interaction with the aforesaid ChE-inhibitors.

Binding studies have demonstrated that tacrine and to a lesser degree eserine, neostigmine and edrophonium, all displace [^{3}H]TCP (N-[1-(2-thienyl)cyclohexyl]-3,4-[^{3}H]piperidine), a radioligand specific for the PCP site, in rat brain homogenates (Albin *et al.,* 1988). Clinical studies have also shown that tacrine reduces the duration of anaesthesia and emergence symptoms associated with dissociative anaesthetics such as PCP and ketamine, suggesting an antagonistic effect of tacrine (Albin *et al.,* 1974). However observations made from *in vitro* studies using murine cortical cultures, suggest that the neuroprotective effects of tacrine against the NMDA-mediated toxicity, may possibly be due to a PCP-like action, resulting in the blockade of the NMDA channel (Davenport *et al.,* 1988). The cholinesterase inhibitors eserine, neostigmine and edrophonium also showed partial neuroprotection at similar concentrations in the same study. Furthermore PCP, ketamine, MK-801 and also the 'σ opiates' N-allylnormetazocine (SKF 10047 or NANM) and cyclazocine have all been found to share the ability of antagonizing the neuroexcitatory effects of NMDA on central neurons (Anis *et al.,* 1983; Davenport *et al.,* 1988; Berry *et al.,* 1984). It

has been suggested that tacrine may be exerting some of its clinical effects through a mixed agonist-antagonist interaction at the PCP site (Davenport *et al.,* 1988).

It is now well known that the PCP site is part of the NMDA receptor complex and it has also been shown that PCP-type ligands antagonize the neuroexcitatory effects of NMDA on central neurons possibly by blocking the open NMDA channel (cited in Davenport *et al.,* 1988). However the neurochemical mechanisms of action of PCP are widespread and it has also been shown to interact with dopaminergic and cholinergic systems. PCP-induced stereotypy, turning behaviour and locomotor activity indicate that PCP may act by indirectly releasing dopamine (Leventer & Johnson, 1983). At a biochemical level PCP has been shown to inhibit competitively, both butyryl and acetylChE (Leventer & Johnson, 1983). PCP has also been shown to antagonize the action of ACh at muscarinic receptors in the guinea pig ileum (Leventer & Johnson, 1983) and also to displace the nicotinic channel blocker [^{3}H]perhydrohistrionicotoxin ([^{3}H]H_{12}-HTX) (Eldefrawi *et al.,* 1981). Furthermore PCP has also been shown to affect ACh release from different areas of the brain. There is evidence that it inhibits the K^+-evoked [^{3}H]ACh release in the striatum (Leventer & Johnson, 1983) and others have reported that it has no effect (Junien *et al.,* 1991) and also that it potentiates (Jackisch *et al.,* 1987) depolarization-evoked [^{3}H]ACh release in the hippocampus.

Until recently, PCP sites were thought to be identical to σ binding sites, based on a number of findings. For example benzomorphans such as NANM, cyclazocine and pentazocine, in addition to their antagonist activity at the NMDA receptor complex, were also shown to induce psychotomimetic effects similar to PCP in humans (cited in Campbell *et al.*, 1989; Haertzen, 1970). Further behavioural studies demonstrated that PCP generalized to (+)NANM in drug discrimination tests (Holtzman, 1982; Shannon, 1982) and radioligand binding studies showed that the pharmacological specificity of [^{3}H]PCP and [^{3}H](+)NANM binding sites was similar (Mendelsohn *et al.,*

1985). Hence the single receptor site for PCP analogs and psychotomimetic benzomorphans was designated the 'σ/PCP' receptor.

The existence of σ binding sites was first proposed following the observation that opiate benzomorphans induced unique behavioural effects in the chronic spinal dog. Martin and co workers reported that benzomorphans such as NANM, cyclazocine and pentazocine, induced a characteristic pattern of 'canine delirium' identified by stereotyped head movements, nystagmus, vocalization and restlessness which distinguished these compounds from morphine-like and ketocyclazocine-like opioids (Martin *et al.,* 1976; Gilbert & Martin, 1976). Moreover it was also observed that the effects of these drugs showed stereoselectivity that was the reverse of the classical opiate effects and were not abolished by the opiate antagonist naloxone (Brady *et al.,* 1982). Further studies indicated that the psychotomimetic effects elicited by the benzomorphans were similar to those induced by PCP (Brady *et al.,* 1982; Brent, 1991; Vaupel, 1983).

Evidence for the existence of separate PCP and σ sites was provided by autoradiographic and ligand binding studies (Largent *et al.,* 1984a). It was shown by comparison of the rank order potencies of several drugs for the inhibition of the binding of (+)[^{3}H]NANM and [^{3}H]TCP, that these sites were considerably different. Moreover the drug selectivity pattern of [^{3}H]NANM was observed to differ from that of [^{3}H]PCP showing that these drugs bind to different receptors. For example certain antipsychotics such as haloperidol were shown to displace [^{3}H]NANM with high affinity whereas haloperidol was inactive against [^{3}H]PCP binding; conversely PCP was shown to be weak against [^{3}H]haloperidol binding (Walker *et al.,* 1990). It was clear from these studies that [^{3}H]NANM binds at two distinct sites; a haloperidol sensitive site known as the σ site and a PCP site.

With the development of selective σ ligands such as (+)-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine ((+)-3-PPP) (Largent *et al.,* 1984b) and 1,3-di-o-tolylguanidine (DTG) (Weber *et al.,* 1986) which have negligible affinity for the PCP binding site, identification of distinct σ binding sites was made possible in brain tissue thus distinguishing the σ and the PCP binding site as distinct physical entities. Largent *et al.,* (1986) described a cell line (NCB-20 hybrid neurotumor cells) which is labelled by (+)[^{3}H]NANM and (+)[^{3}H]3-PPP but not by [^{3}H]PCP. More evidence has accumulated from drug discrimination tests in which PCP failed completely to generalize to (+)-pentazocine (Steinfels *et al.,* 1988) and also from ontogenic studies showing divergent postnatal development of σ and PCP binding sites (Majewska *et al.,* 1989). Thus it is now widely accepted that σ binding sites are distinct from PCP receptors. The σ binding site is currently defined as a non opioid, binding site exhibiting a high affinity for haloperidol, DTG, (+)3-PPP and (+)NANM (Quirion *et al.,* 1987).

Autoradiographic studies of rat and guinea pig brain have shown that the distribution of σ binding sites is distinct from any other known neurotransmitter receptor. Preferential localization has been observed, of (+)-[^{3}H]3-PPP binding sites in the pyramidal cells of the hippocampus, hypothalamus, choroid plexus, the ganglia of the ponticular region and the purkinje cells of the cerebellum (Gundlach *et al.,* 1986b). [^{3}H]DTG binding sites are also concentrated in the limbic and sensory-motor structures (Weber *et al.,* 1986). Areas in the forebrain include the diagonal band of Broca, the septum, the paraventricular nucleus of the hypothalamus, the anterodorsal thalamic nucleus, the zona incerta and the reticulum of the thalamus. Sigma binding sites are also found in the midbrain (the dorsal raphe and the substantia nigra) and in the hind brain (the locus coeruleus) (Weber *et al.,* 1986). In addition to its distribution in the brain, σ binding sites have also been found in endocrine organs such as the pituitary, adrenal, testis, ovary (Wolfe *et al.,* 1989) and also in the rat heart (Dumont & Lemaire, 1991), guinea pig ileum (Roman *et al.,* 1988) and vas deferens (Vaupel &

Su, 1987) and at high concentrations in the membrane fractions of the spleen, liver and on lymphocytes (Itzhak *et al.*, 1990 & 1991). Furthermore the subcellular distribution of the σ binding sites appears to be different from typical neurotransmitter receptors - a high concentration of σ binding sites have been found in the plasma membrane and also in the mitochondrial and microsomal fractions of cell homogenates (Itzhak *et al.*, 1991).

Sigma activity has been implicated in numerous studies of all types. Several bioassays have been employed in order to define a physiological function for these σ binding sites. Sigma modulation of smooth muscle contraction has been observed in the guinea pig ileum where σ binding sites have been described in the longitudinal muscle/ myenteric plexus (LMPP) preparation (Campbell *et al.*, 1989). DTG and its congeners inhibited contractions evoked by electrical stimulation or by 5-HT in a manner which correlated well with their binding affinities. This inhibition of evoked contractions was suggested to be due to the inhibition of ACh release and not a direct effect on muscarinic receptors in the LMMP preparation (Campbell *et al.*, 1991). (+)NANM, however was shown to have the opposite effect of potentiating the stimulated contractions in the same preparation (Campbell *et al.*, 1989) - the reason for this remains unclear. Electrophysiological studies have demonstrated an inhibition of K^+ currents by σ ligands (Wu *et al.*, 1990). Data obtained from single cell voltage clamp studies performed on NCB-20 cells were shown to display an excellent correlation with the binding affinities observed in the same cells as well as those obtained previously in the isolated guinea pig vas deferens assay (Vaupel & Su, 1987), where σ ligands have been shown to potentiate electrically induced contractions.

Biochemical studies have shown σ ligands to have an inhibitory effect on carbachol- and noradrenaline-stimulated phosphatidylinositol metabolism (Candura *et al.*, 1990). Although some σ ligands have low affinity for muscarinic receptors, there is evidence

for the involvement of σ binding sites in inositolphosphate turnover. Sigma ligands show reduced ability to inhibit the oxotremorine-M stimulated inositolphosphate response in three σ1–deficient cell lines, indicating the relevance of σ1 sites in this inhibitory mechanism (Bowen *et al.,* 1991). Sigma ligands have also been shown to reverse the increases in cGMP mediated by the NMDA receptor complex (Rao *et al.,* 1991).

Behavioural studies demonstrate that σ binding sites also appear to play a role in the regulation of movement and posture. Microinjection of various σ ligands into the red nucleus of rats have been shown to produce marked alterations in posture and tone (Walker *et al.,* 1990) resembling the motor alterations observed in humans following neuroleptic treatment. The potency of these σ ligands in producing postural effects was highly correlated with their affinity for σ receptors. Also microinjection of σ ligands in the substantia nigra was shown to produce contralateral circling, an effect eliminated by 6-hydroxydopamine lesions of nigral cells (Walker *et al.,* 1990). DTG was shown to be the most potent in producing this effect. Benzomorphan σ ligands have also been shown to stimulate dopamine synthesis in the rat corpus striatum (Booth & Baldessarini, 1991). These findings implicate modulation by σ binding sites of central dopaminergic function and is supported by the localization of σ binding sites in these regions of the brain (Weber *et al.,* 1986).

The role of σ ligands in memory processes has also been investigated. The interaction between NMDA receptors and σ binding sites (discussed below) however, may be of considerable importance in such studies as it is known that NMDA antagonists have amnesic effects on learning and memory mechanisms. Studies demonstrating the effect of the selective σ ligand JO1784 have shown it to reverse the amnesic effects of scopolamine in the passive avoidance task in rats and it has been suggested that this response may be due to an interaction with the NMDA receptors (Earley *et al.,* 1991).

Several lines of evidence suggest a functional modulation by σ ligands, of the events mediated by the NMDA receptor complex . These include :

- The neuroprotective action of σ ligands such as dextromethorphan, dextrorphan, BMY-14802, opipramol, ifenprodil and its structural analog SL-82.0715 (Rao *et al.,* 1991).
- Modulation of NMDA-induced activation of CA3 dorsal hippocampal pyramidal neurons by σ ligands (Monnet *et al.,* 1991a).
- Reversal of the σ ligand-mediated neuroendocrine and dopamine (DA) turnover effects by the competitive NMDA antagonist CPP [3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid] (Iyengar *et al.,* 1991).
- Attenuation by σ ligands such as ifenprodil, SL-82.0715 and BMY-14802 of responses mediated by the cGMP-dependent NMDA/glycine/PCP/ion channel complex (Rao *et al.,* 1991).
- The inhibition of ischemia-induced glutamate release by σ ligands (Rao *et al.,* 1991).
- The modulation by σ ligands of NMDA-evoked [^{3}H]noradrenaline release from hippocampal slices (Monnet *et al.,* 1991b) and NMDA-induced DA release from the striatum (Rao *et al.,* 1991).

Thus the evidence provided so far seems to suggest that a major physiological role of σ binding sites might be to modulate the function of the NMDA receptor complex. For this reason the experimental work described in the present chapter included examination of potential NMDA receptor effects.

Progress in understanding the function of these σ sites has been hampered by the lack of selective ligands. Initial studies using the "prototypic" σ ligand (+)[^{3}H]NANM showed that it has a high affinity for the haloperidol binding site while also displaying an affinity for the PCP site. 3-PPP, which has a high affinity for the σ sites also displays dopaminergic actions in intact animals. However autoradiography studies

have demonstrated that the distribution of (+)3-PPP binding sites in mammalian brain is distinct from that of the dopamine receptors and very similar to the haloperidol-sensitive (+)NANM binding site (Gundlach *et al.,* 1986). The lack of generalization of (+)3-PPP to dopamine ligands in drug discrimination studies further substantiates its usefulness as a σ ligand (cited in Iyengar *et al.,* 1991).

Recent studies have indicated that DTG labels binding sites distinct from those labelled with (+)3-PPP suggesting the existence of heterogenous populations of σ binding sites (Itzhak & Stein, 1990). There is growing evidence that (+)benzomorphans differentiate the recently identified σ2 from the original σ1 binding site (Hellewell & Bowen, 1990). Sigma1 sites are thought to be those sites highly sensitive to (+)benzomorphans while the σ2 site, shows reversed stereoselectivity i.e (-)benzomorphans being more potent than (+)benzomorphans. DTG and haloperidol have comparable affinity for both sites whereas (+)3-PPP binds with higher affinity to the σ1 site. Although σ2 sites were initially characterized on PC12 cells and in rat liver, they are very similar to the sites described by Itzhak *et al.,* (1991) in mouse brain, by Rothman *et al.,* (1990) in guinea pig brain and Wu *et al.,* (1990) in NCB-20 cells. It is still not absolutely certain whether the findings described above suggest the existence of σ subtypes or different binding states. However pharmacological studies using electrically stimulated guinea pig ileum preparations support the existence of subtypes, as the differences observed cannot be explained as differences due to agonist/antagonist activity (Campbell *et al.,* 1989; Coccini *et al.*, 1991). Further evidence for the existence of σ subtypes also comes from studies of the interaction between σ binding sites and the NMDA receptor complex where the observed effects appeared to be divided into groups characterized by benzomorphan and non benzomorphan σ ligands (Iyengar *et al.,* 1991).

Although σ activity has been implicated in numerous pharmacological, biochemical and behavioural events, the physiological role of the σ site is not well understood.

Also a variety of structural and pharmacological classes of drugs including benzomorphans, morphinans, antipsychotics (triflupromazine, chlorpromazine), phenylpiperazines, phenethylamines, antidepressants (imipramine), anthistamines, anti-parkinsonian agents, muscarinic antagonists (Hudkins & De Haven-Hudkins, 1991), Ca^{2+} channels blockers (Rothman *et al.,* 1990) and monoamine oxidase inhibitors (Itzhak *et al.,* 1991) have been shown to interact with the σ site. Despite the ambiguity of the role of σ sites, their involvement in psychiatric disorders is supported by recent developments of atypical antipsychotics which have a high affinity for the σ binding sites and may also lack the common side effects observed with such treatment. The high incidence of extrapyramidal side effects and tardive dyskinesia associated with most antipsychotics (D2 antagonists) in clinical use today has inspired the search for novel antipsychotics with high efficacy and without extrapyramidal side effects. Of these novel antipsychotic drugs, three have undergone clinical trials and shown beneficial effects in schizophrenic patients - they are rimcazole, remoxipride and tiospirone (Largent *et al.,* 1988). It is possible that σ binding sites may also have some clinical relevence in other disease states.

The effect of PCP and σ ligands on K^+-evoked [^{3}H]ACh release is investigated in this chapter in relation to the effects observed with the ChE-inhibitors tacrine, eserine, neostigmine and edrophonium.

METHODS

1. Release experiments

Hippocampal prisms were prepared, preloaded with [^{3}H]choline and superfused as described in Chapter 2 - General Methods and Materials.

2. Receptor binding assays

a) [^{3}H]QNB binding experiments

Whole membrane preparations were made from freshly dissected hippocampi and radioligand displacement experiments were carried out as described as in Chapter 3.

b) [^{3}H](+)Pentazocine binding experiments

Preparation of tissue

The membrane preparations were prepared following the procedure described by DeHaven-Hudkins *et al.,* (1993). Brain tissue was obtained from male, Sprague Dawley rats weighing 250-300g. The rats were stunned, cervically dislocated and decapitated. The brain was removed and dissected on ice for the hippocampus. The tissue was homogenized in 10 volumes (w/v) of 0.32M sucrose with a glass-teflon homogenizer at 600 r.p.m., 10 strokes. The homogenate was centrifuged at 900*g* for 10 min at 4°C and the resulting supernatant was collected and centrifuged at 22,000*g* for 20 min at 4°C. The pellet was resuspended in 10 volumes of Tris-HCl buffer (50mM, pH 7.4) incubated at 37°C for 30 min and recentrifuged at 22,000*g* for 20 min at 4°C. Following this, the pellet was resuspended in Tris-HCl buffer and frozen in 5ml aliquots at -70°C.

Receptor binding assay

A modified version of the procedure described by DeHaven-Hudkins *et al.,* (1993) was used. Membrane aliquots were freshly thawed, resuspended in fresh Tris-HCl buffer and stored on ice until use. In binding competition studies, each assay tube

contained 100μl of a solution of [^{3}H](+)pentazocine prepared to give a final concentration of 10nM (7.33x10^5 DPM/ml). The [^{3}H](+)pentazocine solution was prepared by adding 20μl of [^{3}H](+)pentazocine (31.6 μCi/nmol) to a 6 ml volume of Tris-HCl (50mM, pH 7.4) containing 10μM atropine to minimize muscarinic receptor interactions by the radioligand. 100μl samples of various concentrations of the drugs to be tested were added to 500μl of the tissue suspension and 300μl of Tris-HCl buffer to give a final assay volume of 1ml. Non-specific binding was defined as residual binding in the presence of 1μM haloperidol. Following addition of the tissue, the asssay tubes were incubated at 37°C in a shaking water bath for 2½ h. The reaction was terminated by rapid filtration on Whatman GF/B glass fibre filters that were presoaked in a solution of 0.5% polyethyleneimine for at least 1 h prior to use. Filters were washed thrice with 4ml volumes of cold Tris-HCl buffer. Following addition of NE260, the scintillation cocktail, samples were allowed to equilibrate for at least 24 h. The amount of bound radioactivity was determined by liquid scintillation spectrometry using a Beckman LS 233 liquid scintillation counter with an efficiency for tritium of approximately 35%.

MATERIALS

Chemicals

From Semat Technical UK Ltd, St. Albans, Hertfordshire, (Research Biochemicals Incorporated, USA):

MK-801 (dizocilpine maleate), (+)NANM hydrochloride, (+)cyclazocine, (+)3PPP hydrochloride

From Sigma Chemical Co. Ltd., Poole, Dorset, UK:

Phencyclidine hydrochloride, (±)pentazocine hydrochloride, haloperidol, DTG (1,3-di(2-tolyl)guanidine), Tris-HCl, polyethyleneimine.

Radiochemicals

From New England Nuclear (NEN):

[^{3}H](+)Pentazocine in ethanol solution; specific radioactivity 1169 GBq/mmol (31.6 Ci/mmol).

RESULTS

1.The effect of noncompetitive NMDA receptor antagonists on K^+-evoked $[^3H]$ACh release.

MK-801

MK-801 at concentrations ranging from 1-50μM did not affect K^+-evoked $[^3H]$ACh release as shown in Figure 4.1a and Table 4.1 below. Neither did MK-801 (100μM) affect the potentiation of release caused by 100μM neostigmine (Figure 4.1b) - control S2/S1 0.633 ± 0.034 (n=6); 100μM neostigmine S2/S1 1.385 ± 0.055 (n=8); 100μM neostigmine and 100μM MK-801 S2/S1 1.277 ± 0.091 (n=8); P>0.05.

CONCENTRATION MK801	S2/S1 RATIO CONTROL	S2/S1 RATIO MK801
1μM	0.842 ± 0.030 (n=13)	0.796 ± 0.032 (n=16)
10μM	0.842 ± 0.030 (n=13)	0.789 ± 0.043 (n=15)
50μM	0.675 ± 0.025 (n=13)	0.786 ± 0.024 (n=16)

TABLE 4.1

The effect of MK-801 on K^+-evoked $[^3H]$ACh release. The test samples were superfused with MK801 10 min prior to and during the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of the total number of replicates (n) observed from four experiments; see Methods (Chapter 2) for details.

Phencyclidine (PCP)

As shown in Figure 4.2 and Table 4.2 below, phencyclidine at concentrations of 1 and 10μM had no effect on K^+-evoked $[^3H]$ACh release although at 100μM it caused an increase of 17% (P<0.05).

CONCENTRATION PHENCYCLIDINE	S2/S1 RATIO	
	CONTROL	PHENCYCLIDINE
1μM	0.708 ± 0.021 (N=4)	0.731 ± 0.044 (N=4)
10μM	0.738 ± 0.017 (n=16)	0.796 ± 0.023 (n=16)
100μM	0.760 ± 0.028 (n=14)	0.857 ± 0.034* (n=16)

TABLE 4.2

The effect of PCP on K^+-evoked [^{3}H]ACh release. The test samples were superfused with PCP 10 min prior to and during the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) observed from four experiments depending on the analysis of variance; see Methods (Chapter 2) for details. Significant differences with respect to the control are denoted by * $P<0.05$.

2. The effects of the benzomorphan σ ligands

a) The effects of the σ ligand (±)pentazocine on:

K^+-evoked [^{3}H]ACh release

As shown in Figure 4.3, (±)pentazocine does not alter K^+-evoked [^{3}H]ACh release at concentrations 10 and 50μM; control S2/S1 0.681 ± 0.018 n=17; 10μM pentazocine S2/S1 0.724 ± 0.041 n=18; 50μM pentazocine S2/S1 0.613 ± 0.043 n=19; $P>0.05$.

Potentiation of K^+-evoked release of [^{3}H]ACh by ChE-inhibitors

(±)Pentazocine at 50μM concentrations significantly attenuates the potentiation caused by the ChE-inhibitors tacrine, eserine, neostigmine and edrophonium (Table 4.3, Figures 4.4 & 4.5). Concentrations of 10μM however do not affect the potentiation induced by the ChE-inhibitors ($P>0.05$).

CHE-INHIBITOR (CHE-I)	CONTROL	CHE-I	CHE-I + 10μM PENTAZOCINE	CHE-I + 30μM PENTAZOCINE	CHE-I + 50μM PENTAZOCINE
TACRINE (30μM)	_	1.089 ± 0.041 (n=14)	0.972 ± 0.054 (n=16)	_	0.789 ± 0.046** (n=14)
ESERINE (100μM)	_	1.090 ± 0.059 (n=12)	1.161 ± 0.061 (n=15)	_	0.680 ± 0.040** (n=14)
NEOSTIGMINE (100μM)	0.669 ± 0.052 (N=4)	1.212 ± 0.039 (N=8)	1.363 ± 0.082 (N=4)	1.053 ± 0.107 (N=4)	0.887 ± 0.039* (N=4)
EDROPHONIUM (100μM)	_	1.220 ± 0.040 (n=15)	1.316 ± 0.063 (n=16)	_	0.887 ± 0.028** (n=15)

TABLE 4.3

The effect of the benzomorphan σ ligand (±)pentazocine on the ChE-inhibitor-induced potentiation of K^+-evoked [^{3}H]ACh release.

The test samples were superfused with ChE-inhibitor or ChE-inhibitor and (±)pentazocine 10 min prior to and during the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) observed from four experiments, depending on the analysis of variance; see Methods (Chapter 2) for details. Significant differences with respect to the S2/S1 value of the ChE-inhibitor alone are denoted by * $P<0.01$ and ** $P<0.001$.

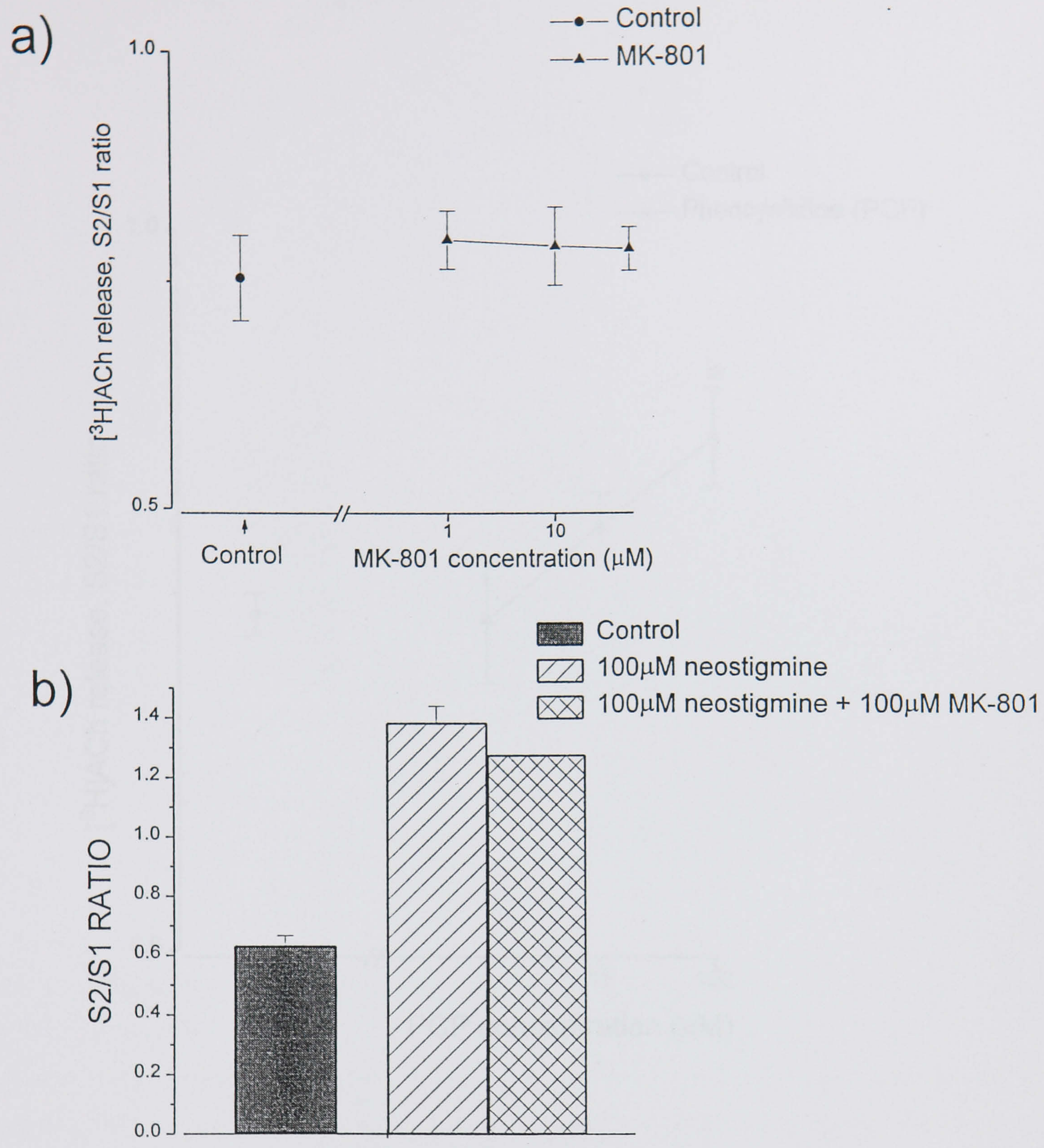


Figure 4.1 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of MK-801.

a) MK-801; 1-50μM concentrations

b) MK-801 in combination with 100μM neostigmine

The prisms were stimulated twice (S1 and S2) for 2 min and MK-801 or MK-801 and neostigmine was added to the superfusing medium 10 min prior to and during the S2 stimulation. The values represent the S2/S1 ratios as the mean ± SEM of the total number of replicate samples (n) observed from 4 (a) and 2 (b) experiments; see Table 4.1 and Results for details.

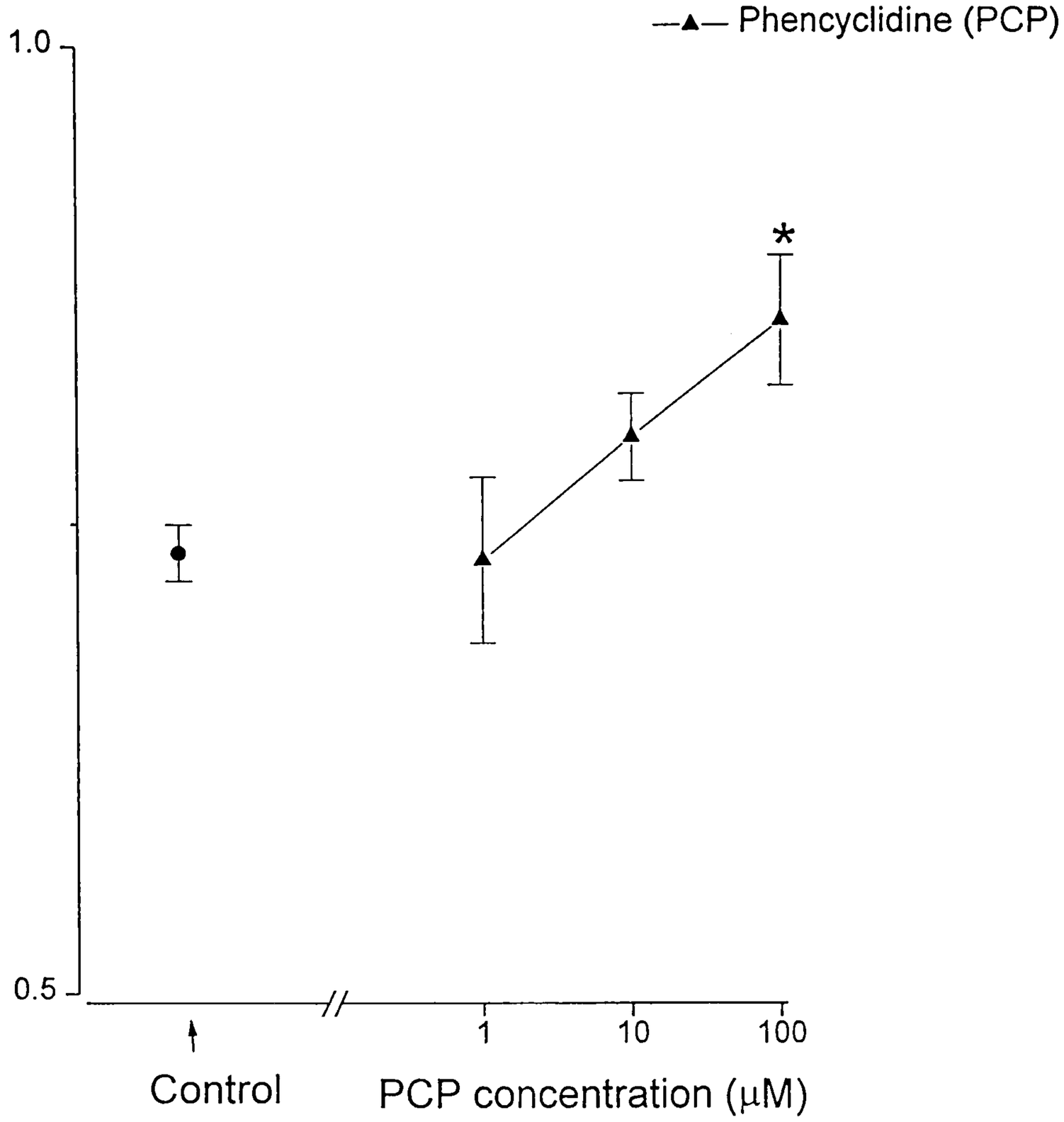


Figure 4.2 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of phencyclidine.

The prisms were stimulated twice (S1 and S2) for 2 min and phencyclidine was added to the superfusing medium 10 min prior to and during the S2 stimulation. Each point represents the S2/S1 ratio as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) observed from 4 experiments; see Table 4.2 for details. The control represents the mean ± SEM of means obtained from all the experiments. Significant differences in the S2/S1 ratio compared to the control group are denoted by * $P<0.05$.

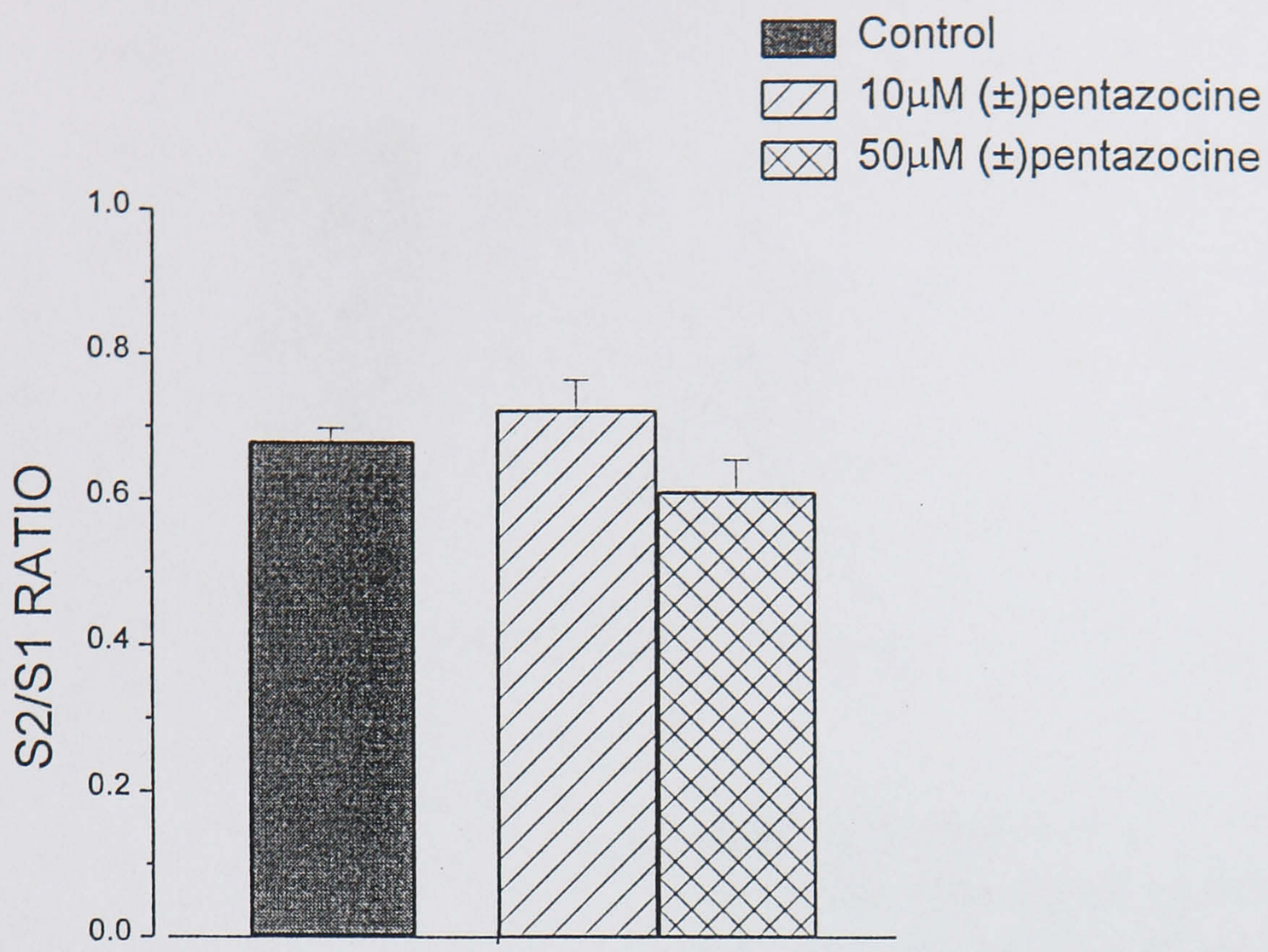


Figure 4.3 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of (±)pentazocine.

The prisms were stimulated twice (S1 and S2) for 2 min and (±)pentazocine was added to the superfusing medium 10 min prior to and during the S2 stimulation. Each column represents the S2/S1 ratio as the mean ± SEM of the total number of replicate samples (n) observed from five experiments; see Results for details.

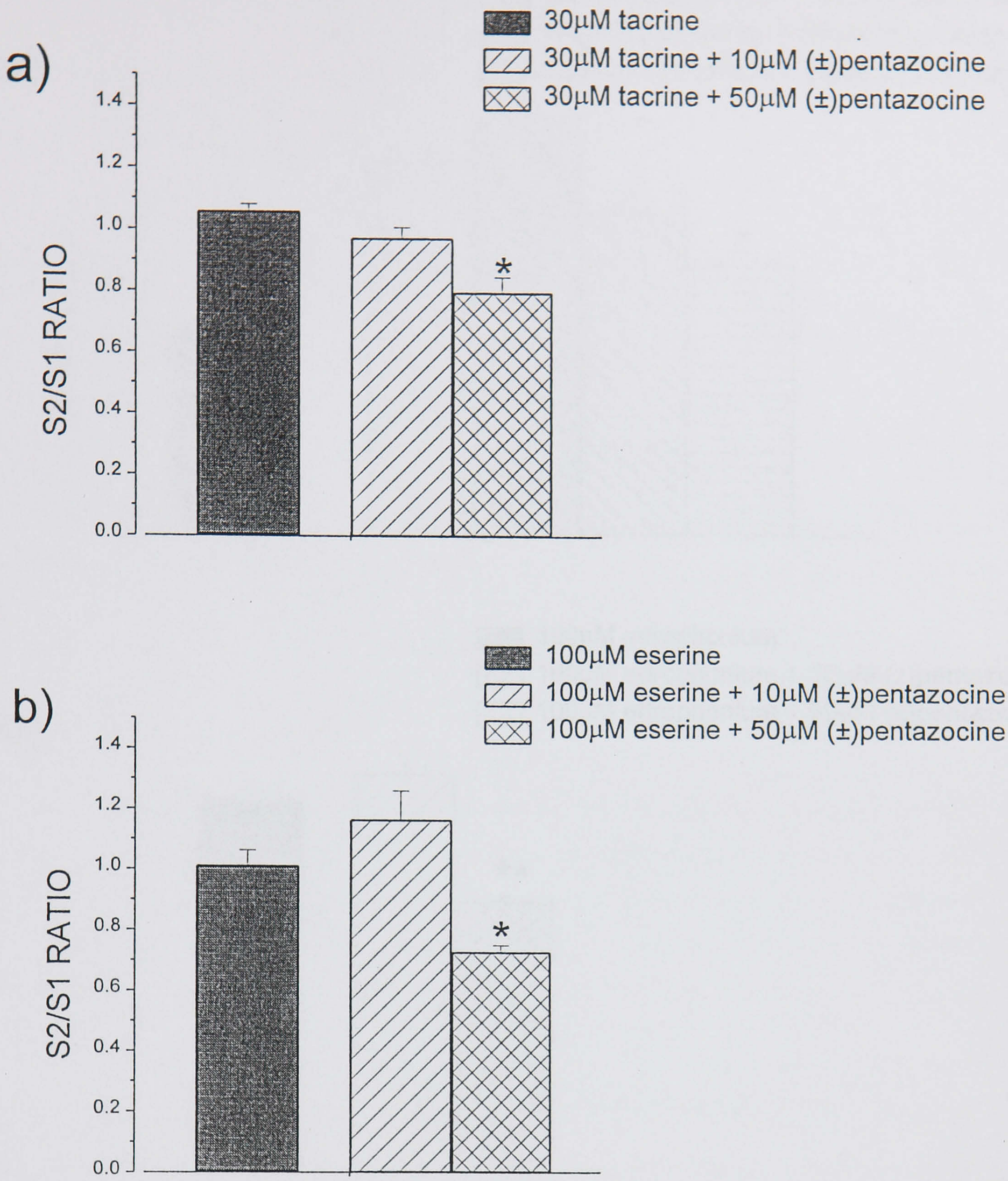


Figure 4.4 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of (±)pentazocine on the potentiation of [^{3}H]ACh release caused by the ChE-inhibitors:

a) 30μM tacrine

b) 100μM eserine.

Each column represents the S2/S1 ratio as the mean ± SEM of the total number of replicate samples (n) observed from four experiments; see Table 4.3 for details. Significant differences in the S2/S1 ratio compared to the ChE-inhibitors alone are denoted by * $P<0.001$.

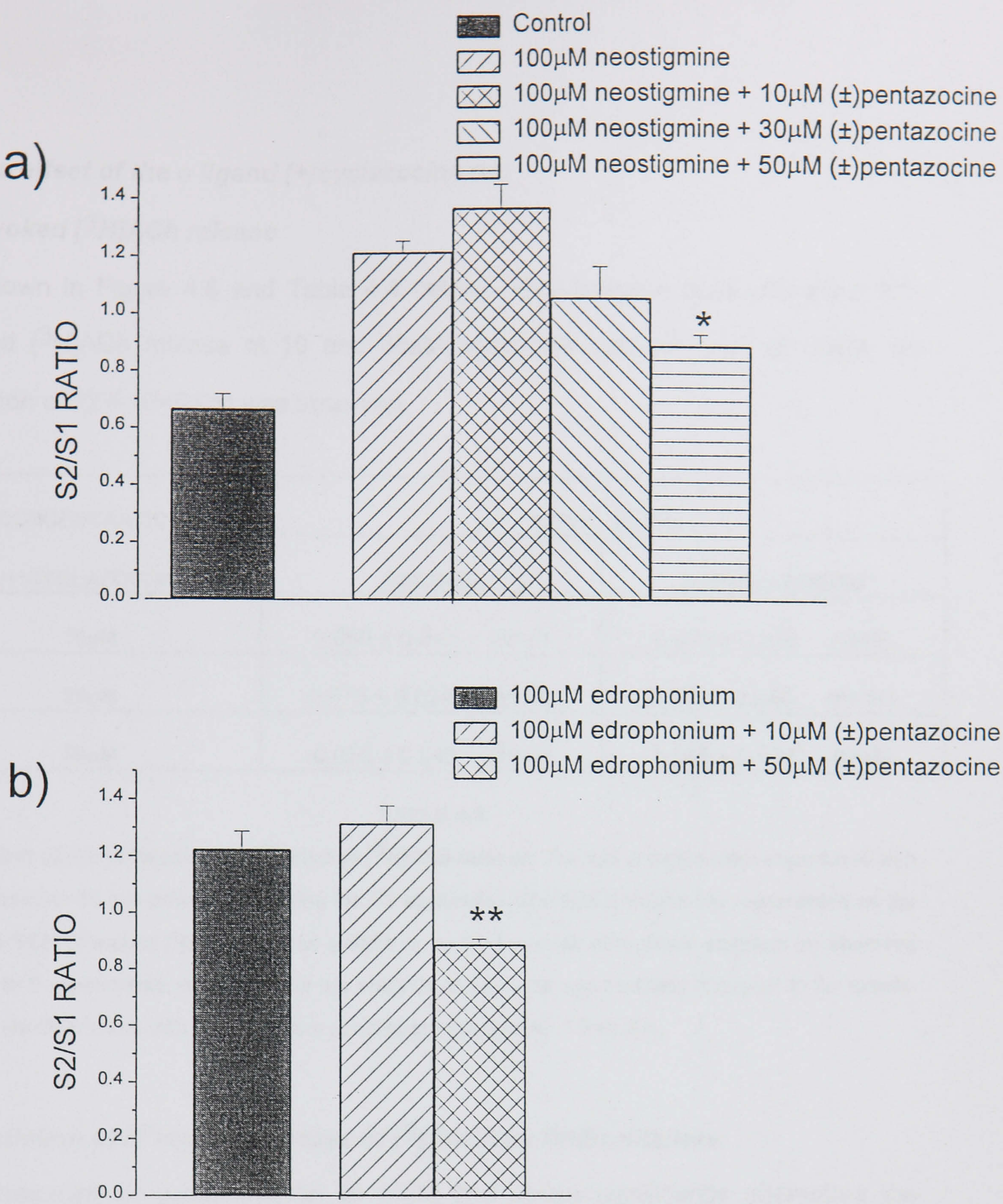


Figure 4.5 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of (±)pentazocine on the potentiation of [^{3}H]ACh release caused by the ChE-inhibitors:

a) 100μM neostigmine

b) 100μM edrophonium.

Each column represents the S2/S1 ratio as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) observed from four experiments; see Table 4.3 for details. Significant differences in the S2/S1 ratio compared to the ChE-inhibitors alone are denoted by * $P<0.01$ and ** $P<0.001$.

b) The effect of the σ ligand (+)cyclazocine on:

K^+-evoked [^{3}H]ACh release

As shown in Figure 4.6 and Table 4.4 below, (+)cyclazocine does not affect K^+-evoked [^{3}H]ACh release at 10 and 30μM concentrations, although at 50μM, an inhibition of 22% ($P<0.05$) was observed.

CONCENTRATION (+)CYCLAZOCINE	S2/S1 RATIO	
	CONTROL	(+)CYCLAZOCINE
10μM	0.669 ± 0.043 (N=5)	0.569 ± 0.054 (N=5)
30μM	0.673 ± 0.024 (n=12)	0.669 ± 0.040 (n=14)
50μM	0.669 ± 0.043 (N=5)	0.525 ± 0.037* (N=5)

TABLE 4.4

The effect of (+)cyclazocine on K^+-evoked [^{3}H]ACh release. The test samples were superfused with (+)cyclazocine 10 min prior to and during the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) observed from 4 to 5 experiments, depending on the analysis of variance; see Methods (Chapter 2) for details. Significant differences with respect to the controls are denoted by * $P<0.001$.

Potentiation of K^+-evoked release of [^{3}H]ACh by ChE-inhibitors

(+)Cyclazocine at concentrations of 10μM and above significantly attenuates the potentiation induced by the ChE-inhibitors tacrine, eserine, neostigmine and edrophonium, although not to control S2/S1 values (Table 4.5, Figures 4.7 & 4.8).

CHE-INHIBITOR (CHE-I)	CONTROL	CHE-I	CHE-I + 2μM CYCLAZOCINE	CHE-I + 10μM CYCLAZOCINE	CHE-I + 30μM CYCLAZOCINE	CHE-I + 50μM CYCLAZOCINE
TACRINE (30μM)	–	1.133 ± 0.060 (n=14)	–	0.948 ± 0.032** (n=15)	0.859 ± 0.044*** (n=16)	–
ESERINE (100μM)	–	1.135 ± 0.032 (N=4)	1.049 ± 0.084 (N=4)	0.962 ± 0.051* (N=4)	–	–
NEOSTIGMINE (100μM)	0.669 ± 0.013 (N=9)	1.397 ± 0.041 (N=12)	1.196 ± 0.102 (N=4)	–	0.939 ±0.044**** (N=4)	0.934± 0.044**** (N=5)
EDROPHONIUM (100μM)	–	1.234 ± 0.045 (n=15)	–	1.151 ± 0.049 (n=13)	0.942 ± 0.051*** (n=15)	–

TABLE 4.5

The effect of the benzomorphan σ ligand (+)cyclazocine on the ChE-inhibitor-induced potentiation of K^+-evoked [^{3}H]ACh release.

The test samples were superfused with ChE-inhibitor or ChE-inhibitor and (+)cyclazocine 10 min prior to and during the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) observed from 4 to 5 experiments, depending on the analysis of variance; see Methods (Chapter 2) for details. Significant differences with respect to the ChE-inhibitor alone are denoted by * P<0.05, ** P<0.01, *** P<0.001 and **** P<0.0001.

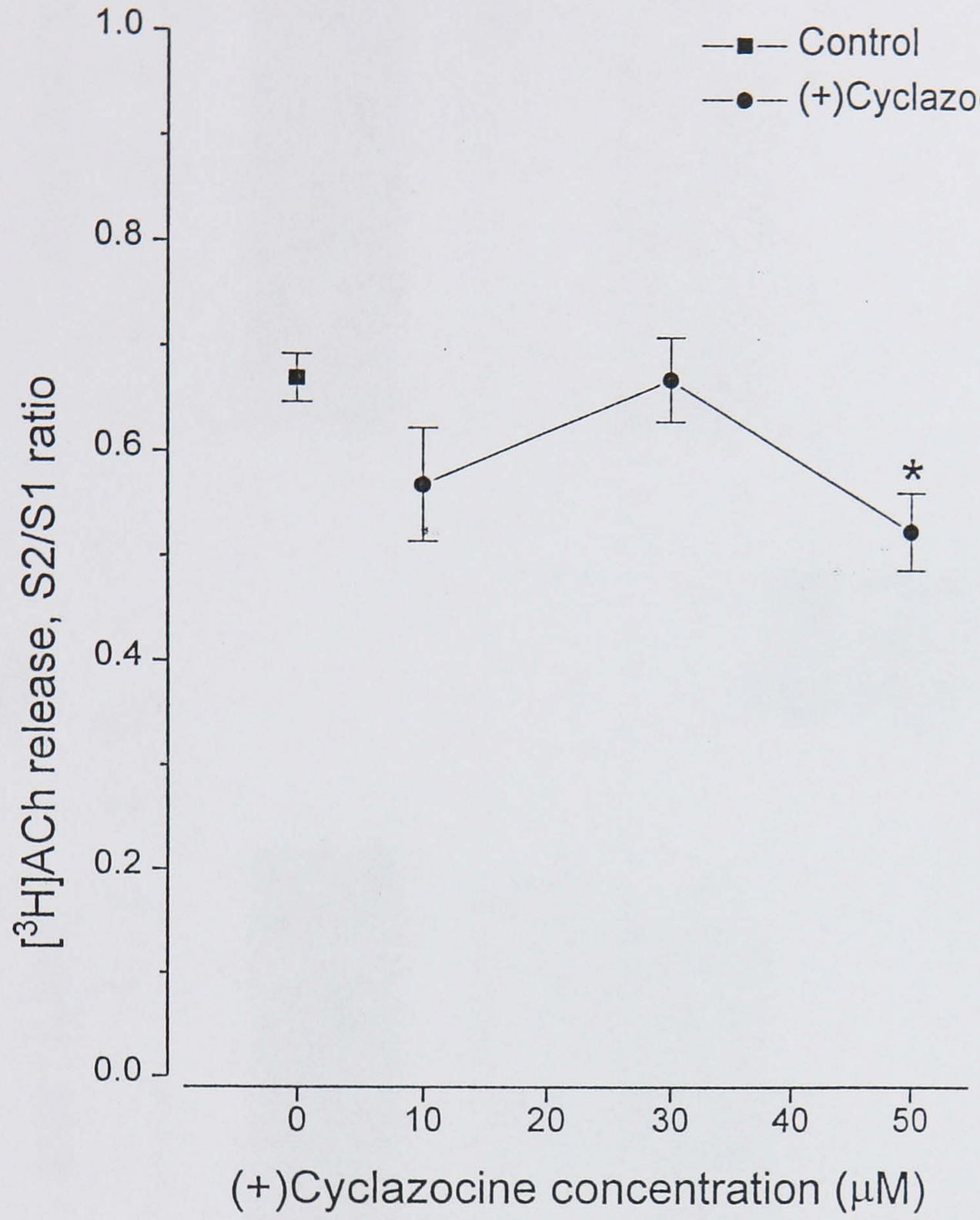


Figure 4.6 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of (+)cyclazocine.

The prisms were stimulated twice (S1 and S2) for 2 min and (+)cyclazocine was added to the superfusing medium 10 min prior to and during the S2 stimulation. Each point represents the S2/S1 ratio as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) observed from 4-5 experiments; see Table 4.4 for details. The control represents the mean ± SEM of the means obtained from all the experiments. Significant differences in the S2/S1 ratio compared to the control group are denoted by * $P<0.001$.

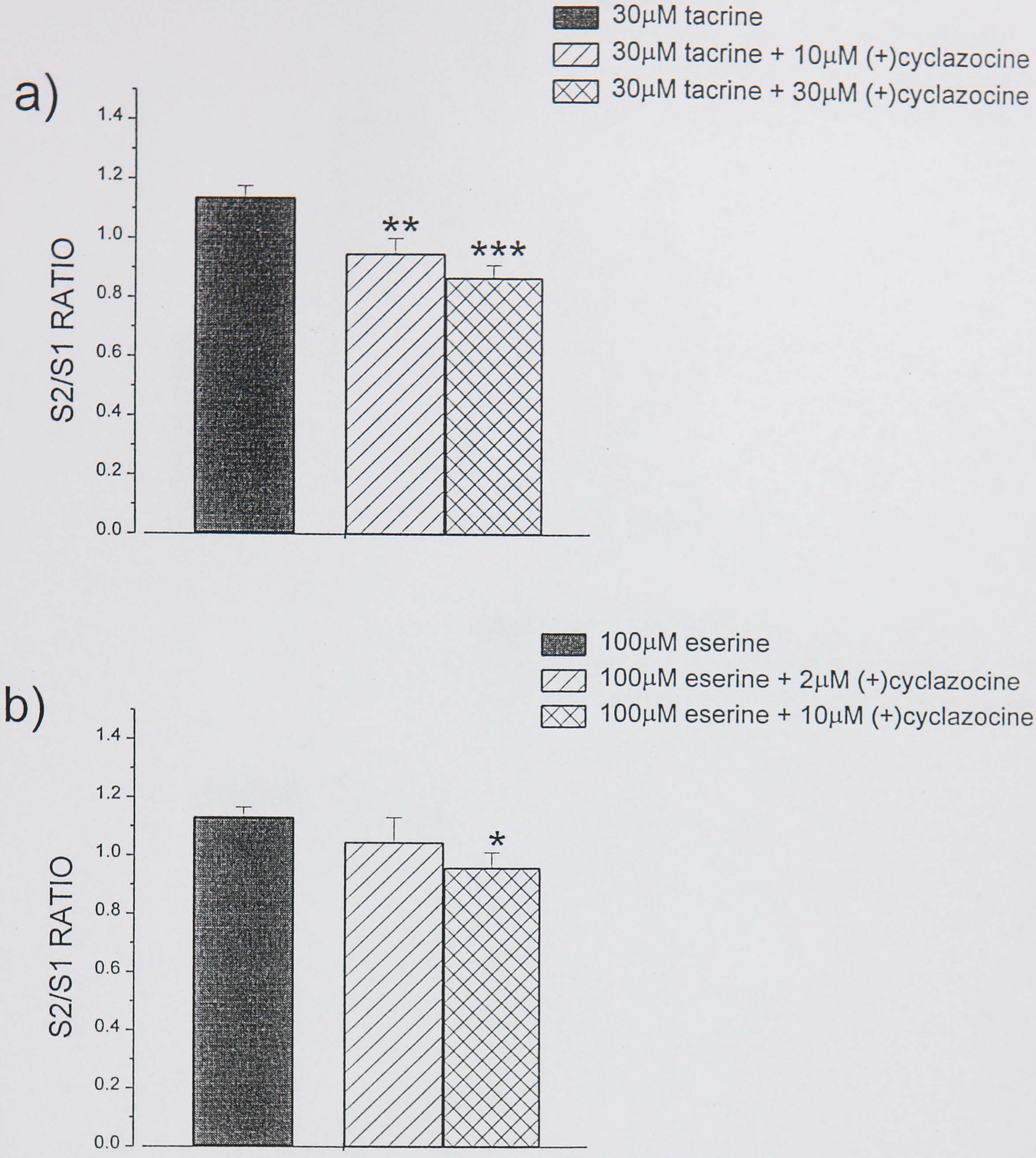


Figure 4.7 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of (+)cyclazocine on the potentiation of [^{3}H]ACh release caused by the ChE-inhibitors:

a) 30μM tacrine

b) 100μM eserine.

Each column represents the S2/S1 ratio as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) observed from four experiments; see Table 4.5 for details. Significant differences in the S2/S1 ratio compared to the ChE-inhibitors alone are denoted by

* $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

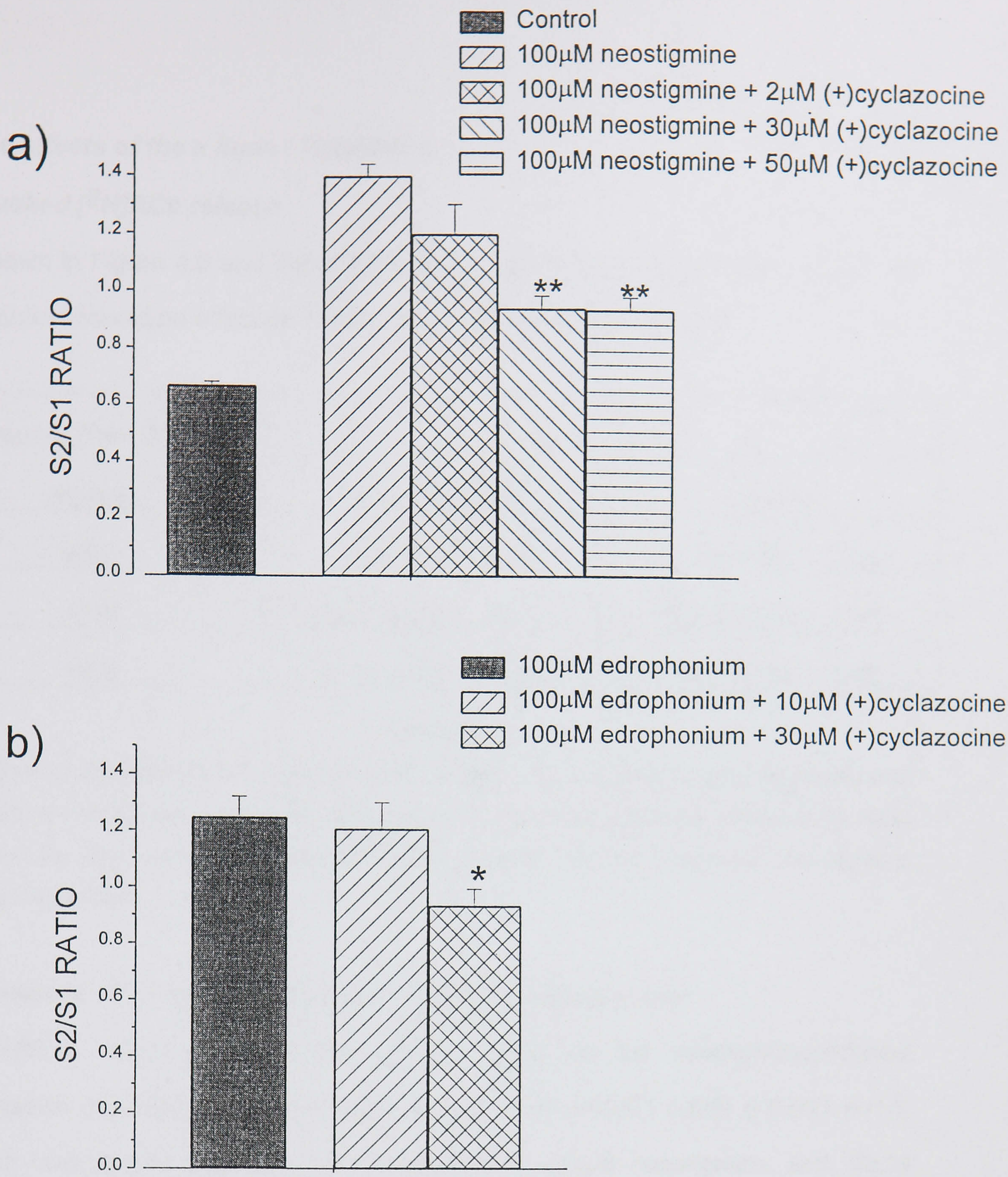


Figure 4.8 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of (+)cyclazocine on the potentiation of [^{3}H]ACh release induced by the ChE-inhibitors:

a) 100μM neostigmine

b) 100μM edrophonium.

Each column represents the S2/S1 ratio as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) observed from four experiments; see Table 4.5 for details. Significant differences in the S2/S1 ratio compared to the ChE-inhibitors alone are denoted by

* $P<0.001$ and ** $P<0.0001$.

c) The effects of the σ ligand (+)NANM on:

K^+-evoked [^{3}H]ACh release

As shown in Figure 4.9 and Table 4.6 below, (+)NANM at concentrations of 0.5, 10 and 30μM showed no effect on K^+-evoked [^{3}H]ACh release; P>0.05.

CONCENTRATION (+)NANM	S2/S1 RATIO CONCENTRATION (+)NANM
0.5μM	0.769 ± 0.019 (n=6) — 0.770 ± 0.040 (n=8)
10μM	0.703 ± 0.037 (n=6) — 0.655 ± 0.048 (n=8)
30μM	0.703 ± 0.037 (n=6) — 0.769 ± 0.077 (n=8)

TABLE 4.6

The effect of (+)NANM on K^+-evoked [^{3}H]ACh release. The test samples were superfused with (+)NANM 10 min prior to and during the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of the total number of replicate samples (n) observed, from two experiments; see Methods (Chapter 2) for details.

Potentiation of K^+-evoked release of [^{3}H]ACh by neostigmine

(+)NANM at 30μM concentrations had no effect on the neostigmine-induced potentiation of [^{3}H]ACh release (Figure 4.10a); control S2/S1 0.669 ± 0.027 n=14; 100μM neostigmine S2/S1 1.245 ± 0.061 n=16; 100μM neostigmine and 30μM (+)NANM S2/S1 1.237 ± 0.061 n=16; P>0.05.

K^+-evoked release of [^{3}H]ACh in combination with naloxone

Naloxone (10μM) had no effect on K^+-evoked [^{3}H]ACh release either alone or in the presence of 10μM (+)NANM (Figure 4.10b); control S2/S1 0.792 ± 0.048 n=7; 10μM naloxone S2/S1 0.661 ± 0.059 n=8; 10μM naloxone and 10μM (+)NANM S2/S1 0.876 ± 0.036 n=8; P>0.05.

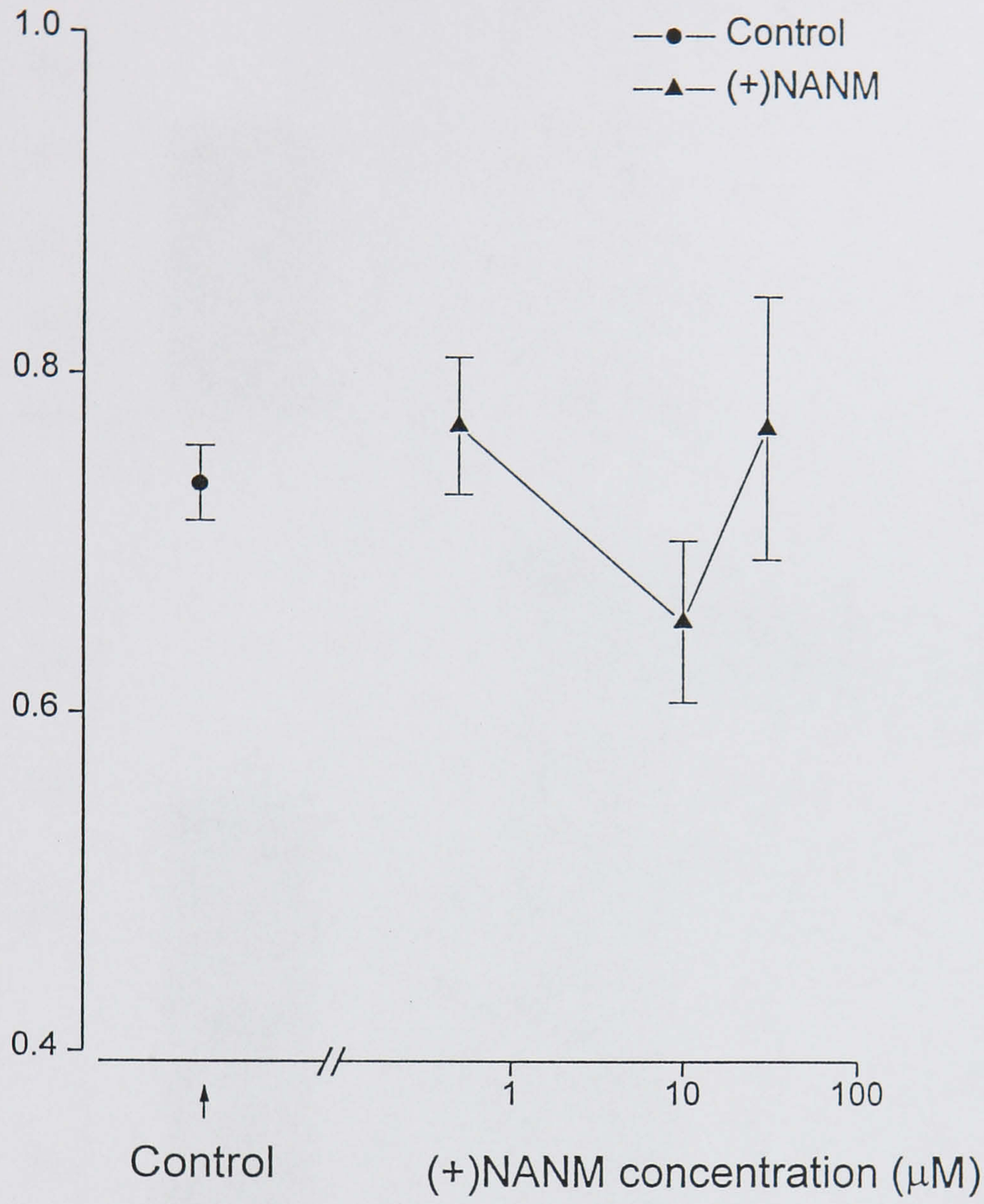


Figure 4.9 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of (+)NANM.

The prisms were stimulated twice (S1 and S2) for 2 min and (+)NANM was added to the superfusing medium 10 min prior to and during the S2 stimulation. Each point represents the S2/S1 ratio as the mean ± SEM of the total number of replicate samples (n) observed from two experiments; see Table 4.6 for details. The control represents the mean ± SEM of means obtained from both experiments.

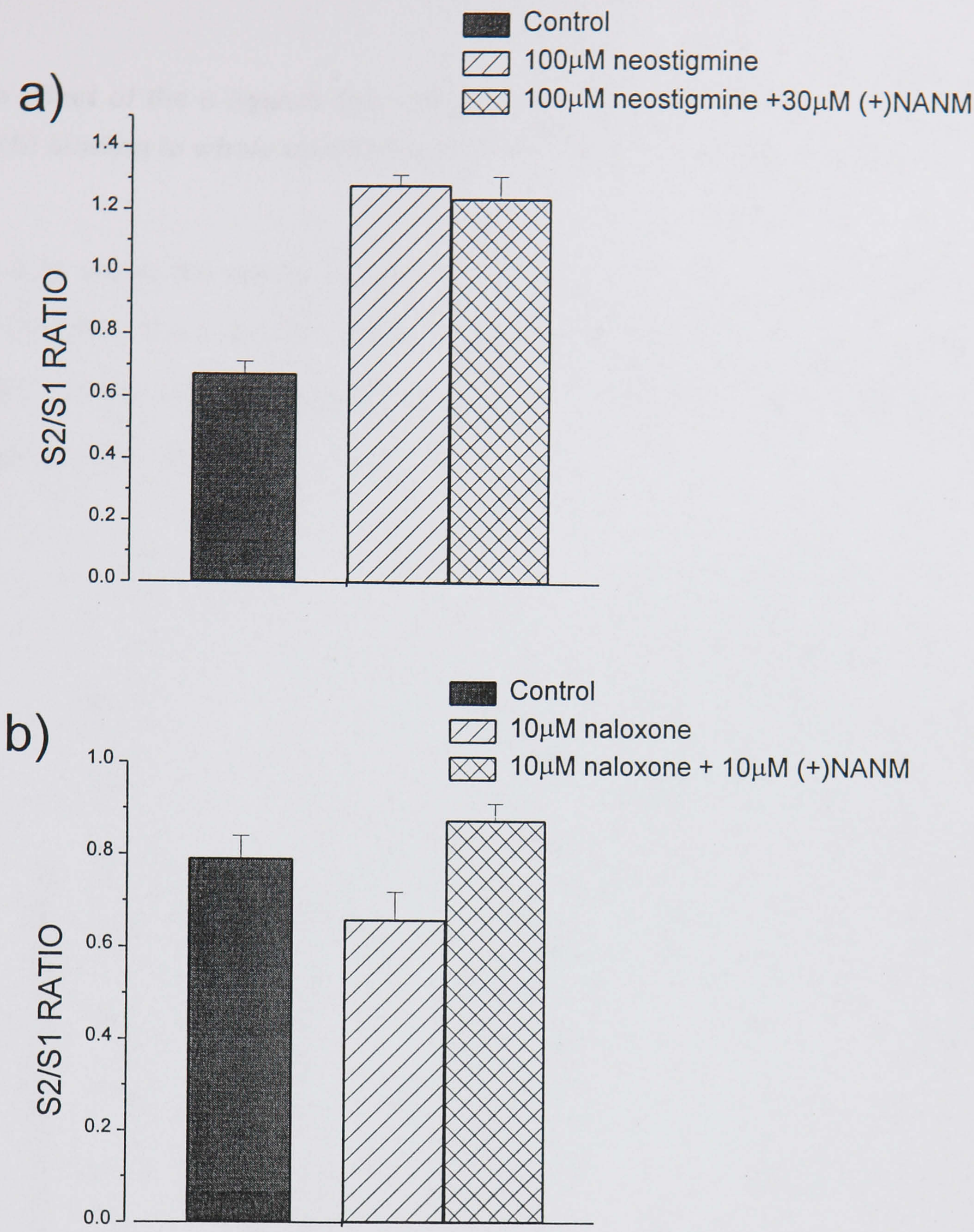


Figure 4.10 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of (+)NANM in combination with:

a) 100μM neostigmine

b) 10μM naloxone

Each column represents the S2/S1 ratio as the mean ± SEM of the total number of replicate samples (n) observed from 2-4 experiments; see Results for details.

d) The effect of the σ ligands (+)cyclazocine, (+)NANM and (±)pentazocine on [^{3}H]QNB binding to whole membrane preparations from rat hippocampus.

Figure 4.11 shows the results of radioligand binding studies using the muscarinic ligand [^{3}H]QNB. The σ ligands (+)cyclazocine, (+)NANM and (±)pentazocine inhibit [^{3}H]QNB binding in a dose-dependent manner - (±)pentazocine is clearly the strongest inhibitor of [^{3}H]QNB binding.

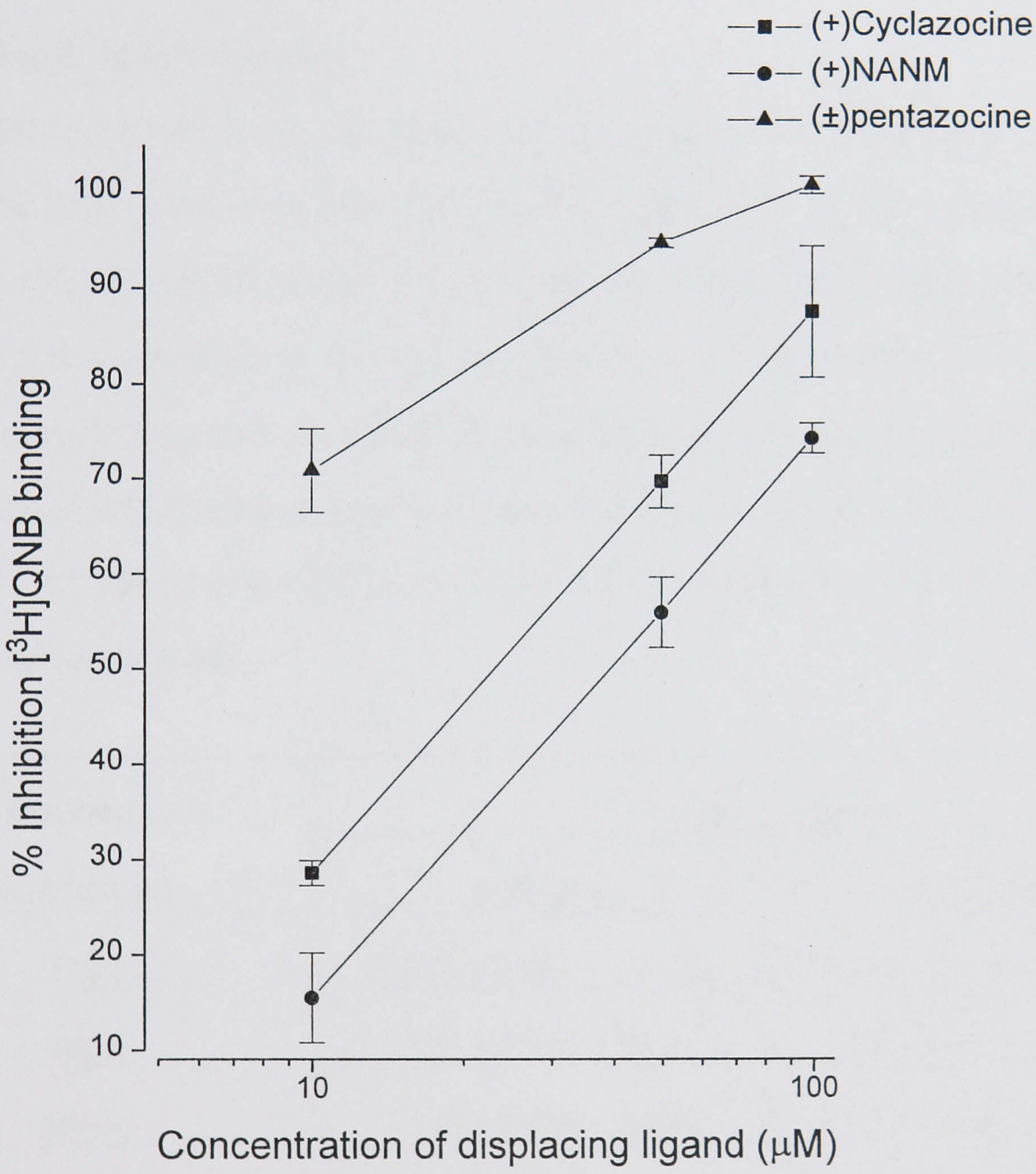


Figure 4.11 Displacement of [^{3}H]QNB binding in rat hippocampal membrane preparations by the σ benzomorphans (±)pentazocine, (+)cyclazocine and (+)NANM.
Each point represents the % inhibition as the mean ± SEM obtained from 2 experiments, each done in quadruplicate.

3. The effects of non benzomorphan σ ligands

a) The effects of the σ ligand haloperidol on:

Potentiation of K^+-evoked release of [^{3}H]ACh by ChE-inhibitors

As shown in Figure 4.12 and Table 4.8, 30μM concentrations of haloperidol produce marked reduction (P<0.001) in the potentiation caused by the ChE-inhibitors tacrine, eserine, neostigmine and edrophonium to control release.

K^+-evoked [^{3}H]ACh release

Haloperidol acting alone also produces marked inhibition of 33 and 53% (P<0.001), at 10 and 30μM concentrations respectively (Figures 4.13 & 4.14 and Table 4.7), while at 100μM concentrations, [^{3}H]ACh release is completely abolished (Figures 4.13a & b & 4.14). Figures 4.13a & b show the time course of the K^+-stimulated release of radiolabel and the effects of haloperidol at the given concentrations. The mean ± SEM S2/S1 ratios corresponding to the data presented in Figure 4.13a & b are 0.665 ± 0.025, 0.274 ± 0.013 and 0.059 ± 0.005 for the controls, 30 and 100μM haloperidol respectively.

CONCENTRATION HALOPERIDOL	S2/S1 RATIO	
	CONTROL	HALOPERIDOL
10μM	0.673 ± 0.024 (n=13)	0.477 ± 0.037* (n=15)
30μM	0.724 ± 0.040 (N=4)	0.326 ± 0.034* (N=4)
100μM	0.724 ± 0.040 (N=4)	0.072 ± 0.011** (N=4)

TABLE 4.7

The effect of haloperidol on K^+-evoked [^{3}H]ACh release. The test samples were superfused with haloperidol 10 min prior to and during the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of means (N) or the means ± SEM of the total number of replicate samples (n) observed from four experiments, depending on the analysis of variance; see Methods (Chapter 2) for details. Significant differences with respect to controls are denoted by * P<0.001 and ** P<0.0001.

CHE-INHIBITOR (CHE-I)	CONTROL	CHE-INHIBITOR	CHE-I + 30μM HALOPERIDOL
TACRINE (30μM)	0.713 ± 0.027 (n=12)	1.154 ± 0.036 (n=15)	0.603 ± 0.026* (n=16)
ESERINE (100μM)	0.662 ± 0.019 (n=13)	1.329 ± 0.078 (n=16)	0.707 ± 0.046* (n=16)
NEOSTIGMINE (100μM)	0.702 ± 0.044 (N=4)	1.284 ± 0.068 (N=4)	0.708 ± 0.115* (N=4)
EDROPHONIUM (100μM)	0.616 ± 0.038 (n=11)	1.139 ± 0.046 (n=16)	0.631 ± 0.046* (n=15)

TABLE 4.8

The effect of the non benzomorphan σ ligand haloperidol on the ChE-inhibitor-induced potentiation of K^+-evoked [^{3}H]ACh release.

The test samples were superfused with ChE-inhibitor or ChE-inhibitor and haloperidol 10 min prior to and during the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) observed from four experiments, depending on the analysis of variance; see Methods (Chapter 2) for details. Significant differences with respect to S2/S1 ratio of the ChE-inhibitor alone are denoted by * $P<0.0001$.

The effects of D1 and D2 dopamine antagonists on the neostigmine-induced potentiation of [^{3}H]ACh release

Sulpiride

Sulpiride, a D2 antagonist had no effect at 30μM concentrations on either the neostigmine-induced potentiation of [^{3}H]ACh release (control S2/S1 0.707 ± 0.041 n=5; 100μM neostigmine S2/S1 1.209 ± 0.058 n=8; 100μM neostigmine and 30μM sulpiride S2/S1 1.293 ± 0.071 n=7; P>0.05; Figure 4.15b) or on K^+-evoked [^{3}H]ACh release (control S2/S1 0.709 ± 0.021 n=7; 30μM sulpiride S2/S1 0.800 ± 0.05 n=8; 100μM sulpiride S2/S1 0.720 ± 0.035 n=8; P>0.05; Figure 4.15a).

SCH 23390

The D1 antagonist SCH 23390 at 30μM concentrations did not alter the effect of neostigmine on K^+-evoked [^{3}H]ACh release (Figure 4.15c); control S2/S1 0.678 ± 0.040 (n=6); 100μM neostigmine S2/S1 1.267 ± 0.057 (n=8); 100μM neostigmine + 30μM SCH 23390 S2/S1 1.264 ± 0.11 (n=8); P>0.05.

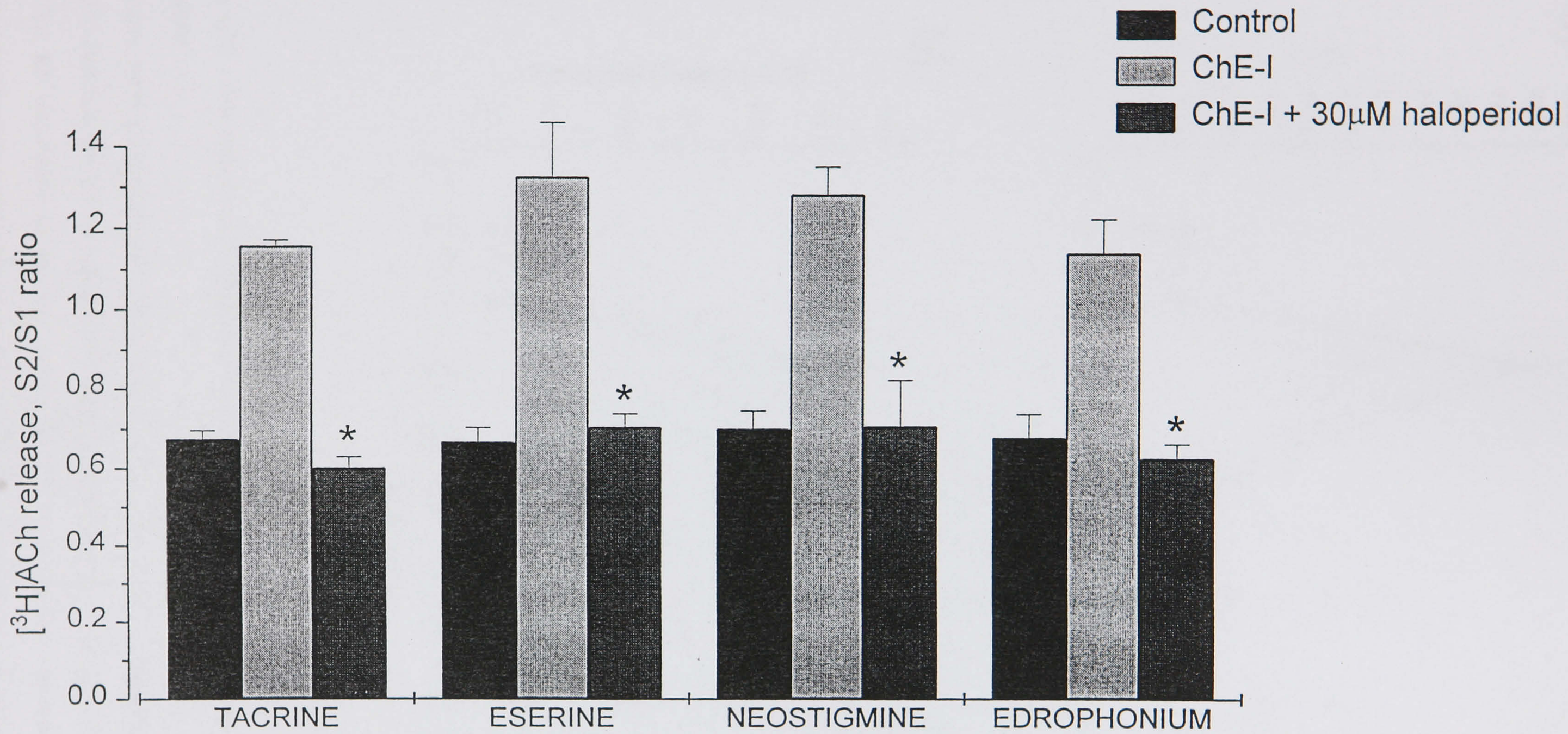
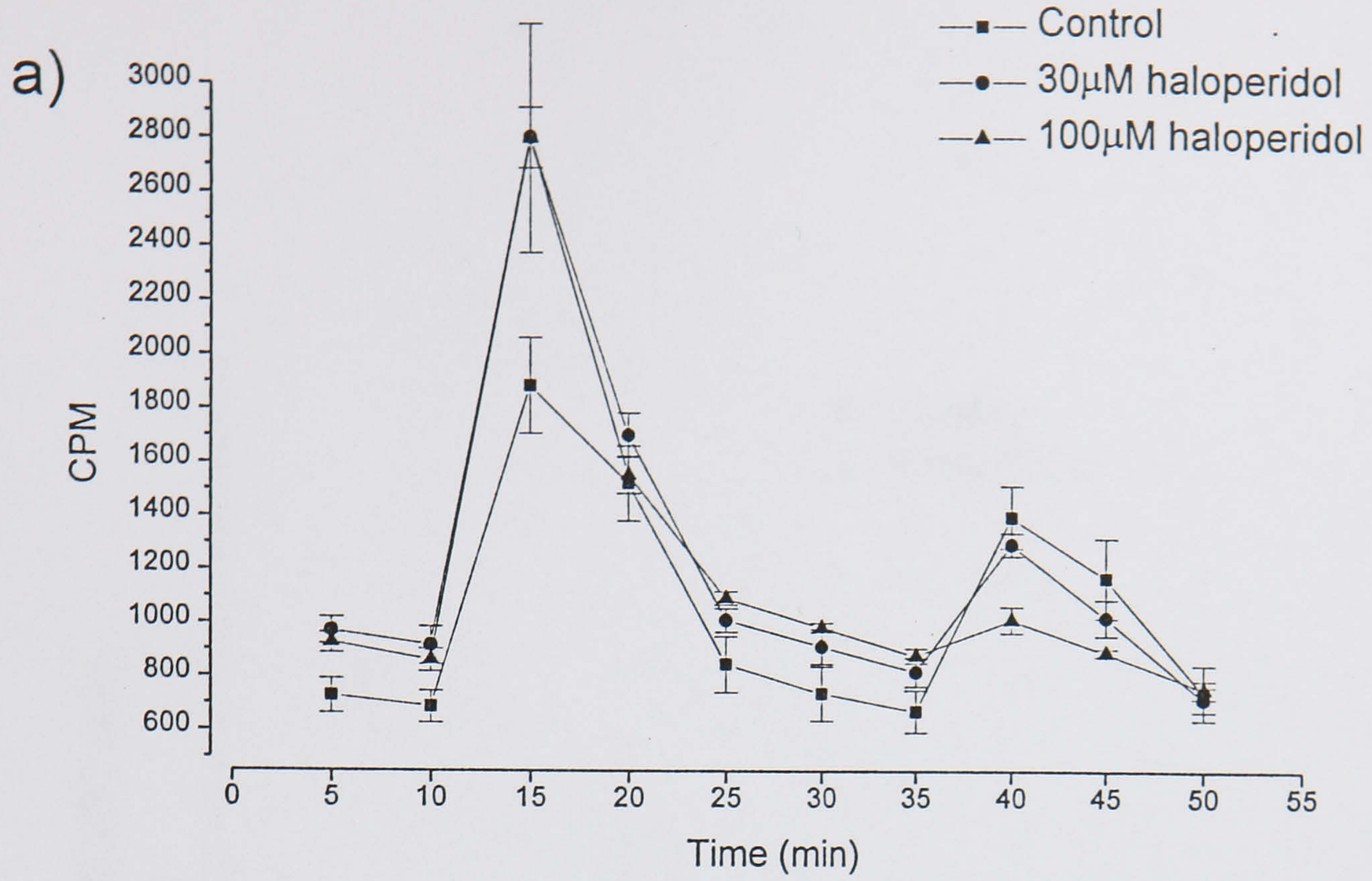


Figure 4.12 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of haloperidol on the potentiation of [^{3}H]ACh release caused by the ChE-inhibitors.

Each column represents the S2/S1 ratio as the mean ± SEM of the means (N) or the mean ± SEM of the total number of replicate samples (n) observed from four experiments; see Table 4.8 for details. Significant differences in the S2/S1 ratio compared to the ChE-inhibitors alone are denoted by * $P<0.0001$.



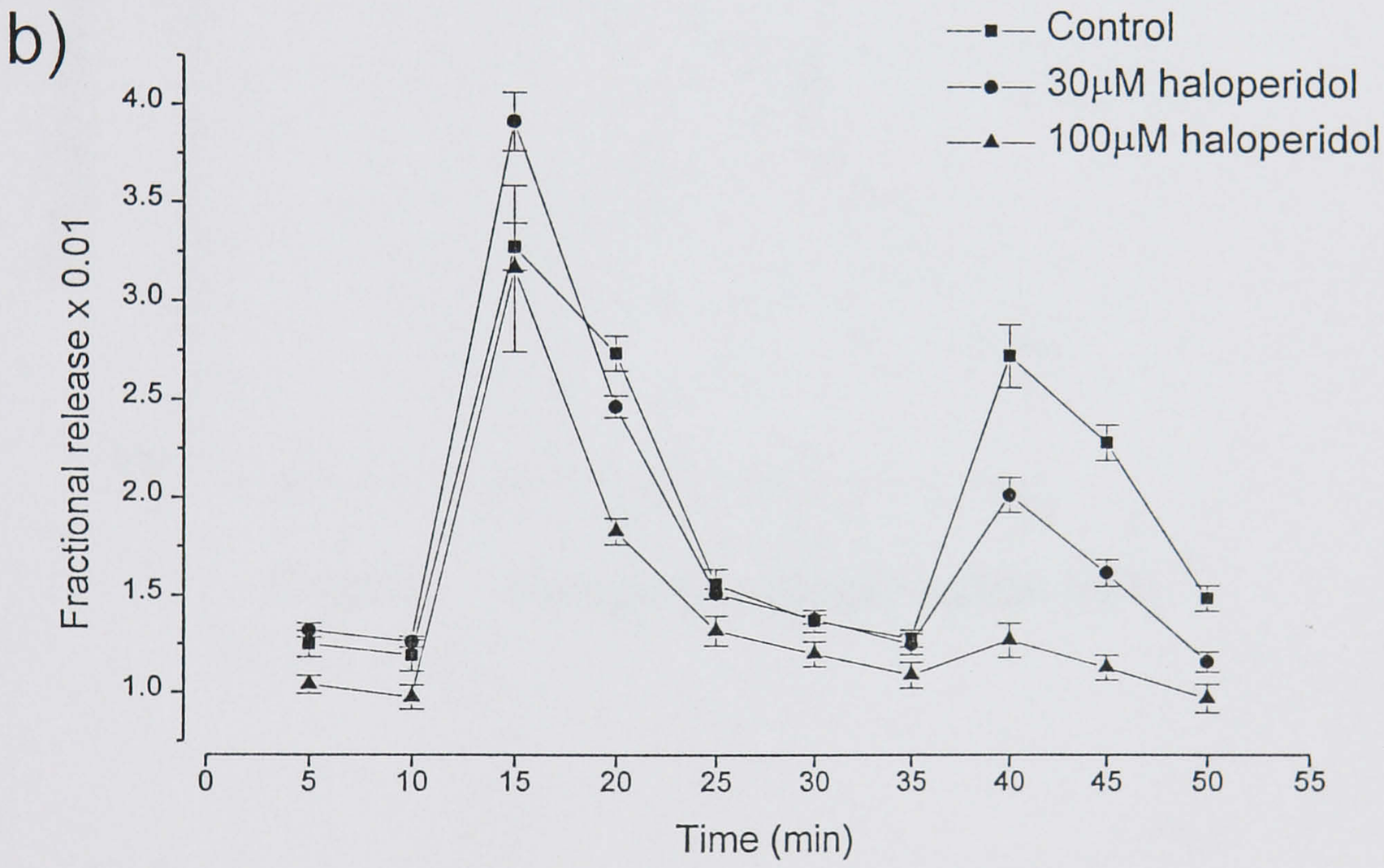


Figure 4.13 the release of radiolabel from hippocampal prisms evoked by 30mM K^+ - the effect of haloperidol.

The prisms were preloaded with [^{3}H]choline (see Chapter 2 for experimental details) and stimulated twice (S1 and S2) for 2 min. Haloperidol was added to the superfusing medium 10 min prior to and during the S2 stimulation in the test group. Each point represents release as the mean ± SEM of replicate samples (per group) observed from one typical experiment and expressed as:

a) CPM

b) fractional release

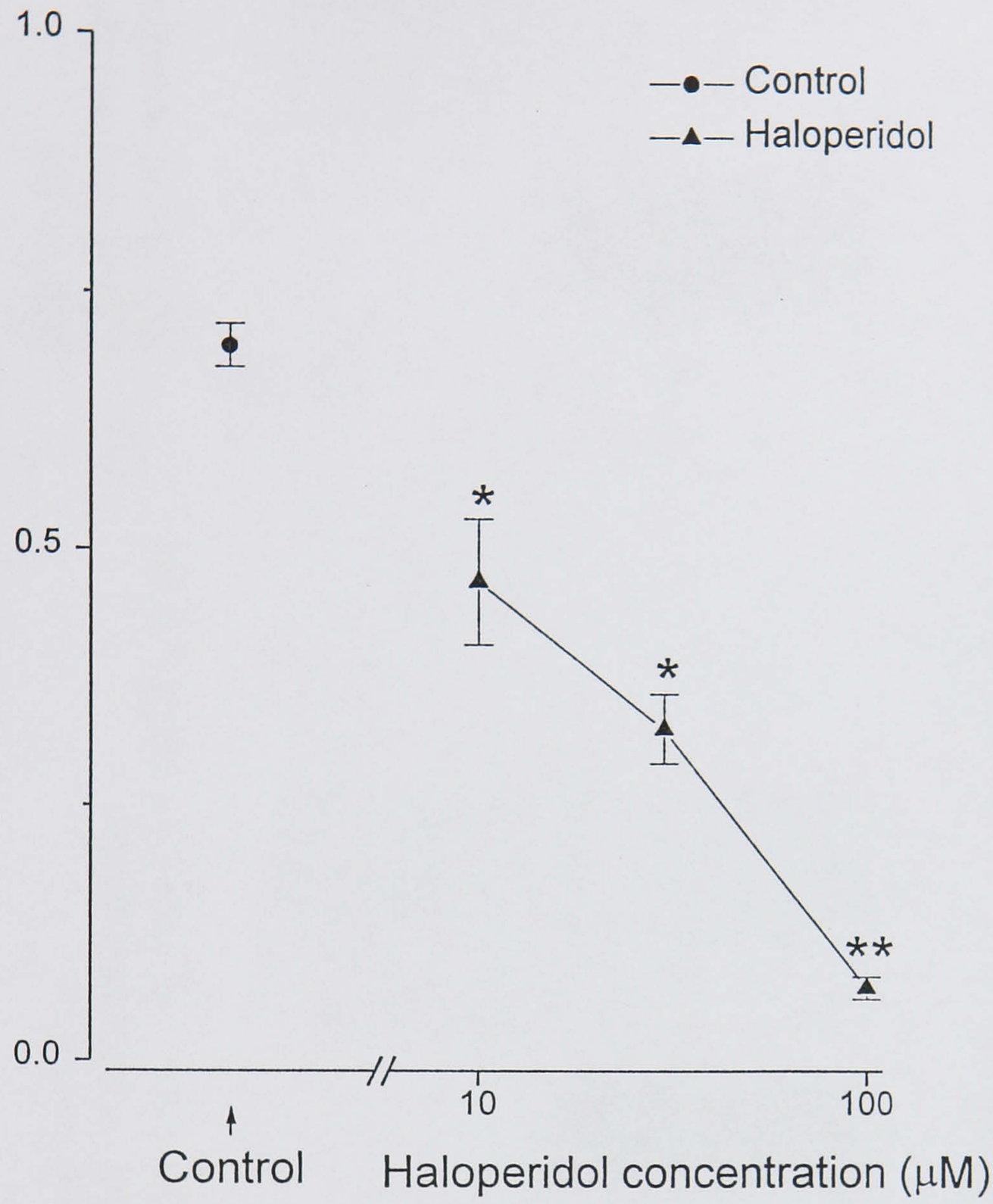


Figure 4.14 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of haloperidol.

The prisms were stimulated twice (S1 and S2) for 2 min and haloperidol was added to the superfusing medium 10 min prior to and during the S2 stimulation. Each point represents the S2/S1 ratio as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) observed from 4 experiments; see Table 4.7 for details. The control represents the mean ± SEM of means obtained from all the experiments. Significant differences in the S2/S1 ratio compared to the control group are denoted by * $P<0.001$ and ** $P<0.0001$.

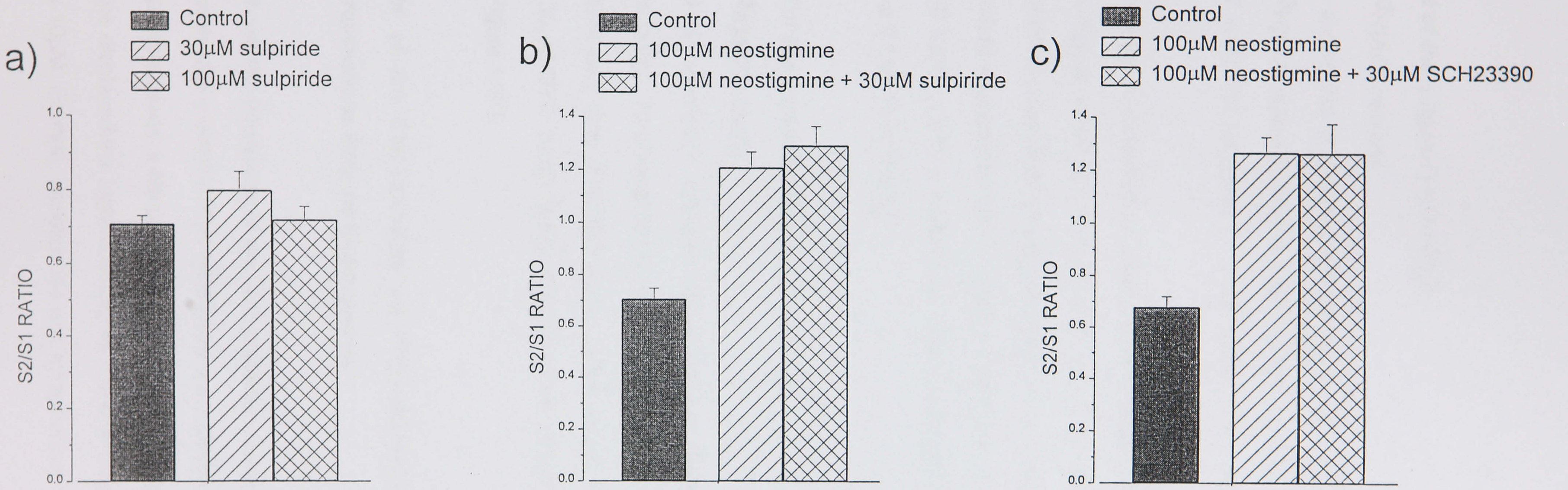


Figure 4.15 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of the dopamine antagonists sulpiride and SCH 23390.

a) the effect of sulpiride alone, on K^+-evoked [^{3}H]ACh release at 30 and 100μM concentrations

b) the effect of 30μM sulpiride on the neostigmine-induced potentiation of K^+-evoked [^{3}H]ACh release

c) the effect of 30μM SCH 23390 on the neostigmine-induced potentiation of K^+-evoked [^{3}H]ACh release

Each column represents the S2/S1 ratios as the mean ± SEM of the total number of replicate samples (n) observed from two experiments; see Results for details.

b) The effect of the σ ligand (+)3PPP on:

K^+-evoked [^{3}H]ACh release

Figure 4.16a shows that (+)3PPP at concentrations 10 and 50μM had no effect on K^+-evoked [^{3}H]ACh release; control S2/S1 0.686 ± 0.034 n=13; 10μM (+)3PPP 0.724 ± 0.037 n=15; 50μM (+)3PPP 0.734 ± 0.043 n=15; P>0.05.

Neostigmine-induced potentiation of K^+-evoked [^{3}H]ACh release

As shown in Figure 4.16b, (+)3PPP had no effect on the neostigmine-induced potentiation of K^+-evoked [^{3}H]ACh release at the above tested concentrations of 10 and 50μM; 100μM neostigmine S2/S1 1.312 ± 0.120 N=4; 100μM neostigmine and 10μM (+)3PPP S2/S1 1.511 ± 0.084 N=4; 100μM neostigmine and 50μM (+)3PPP S2/S 1 1.451 ± 0.119 N=4; P>0.05.

c) The effect of the σ ligand DTG on:

K^+-evoked [^{3}H]ACh release

DTG caused a significant inhibition of K^+-evoked [^{3}H]ACh release at the concentrations tested. At concentrations of 30μM and 100μM release was inhibited by 16% (P<0.05) and 39% (P<0.001) of the control values; control S2/S1 0.682 ± 0.030 n=12; 30μM DTG S2/S1 0.572 ± 0.036 n=14; 100μM DTG S2/S1 0.415 ± 0.040 n=15 (Figure 4.17).

4. The effect of the ChE-inhibitors on [^{3}H]pentazocine binding to whole membrane preparations from rat hippocampus.

Figure 4.18 shows the interaction of the ChE-inhibitors tacrine, eserine, neostigmine and edrophonium with σ binding sites labelled by the σ radioligand [^{3}H]pentazocine. The ChE-inhibitors showed a concentration-dependent inhibition of [^{3}H]pentazocine binding - tacrine displayed the highest affinity for the σ binding sites with an IC_{50} of approximately 10μM. Eserine, neostigmine and edrophonium are weak inhibitors of

[^{3}H]pentazocine binding displaying IC_{50}s greater than 100μM. HC-3 at 100μM concentrations also inhibited the binding of [^{3}H]pentazocine by 16 ± 6%. (+)Cyclazocine and (±)pentazocine at 50μM concentrations caused complete inhibition of [^{3}H]pentazocine binding (data not shown).

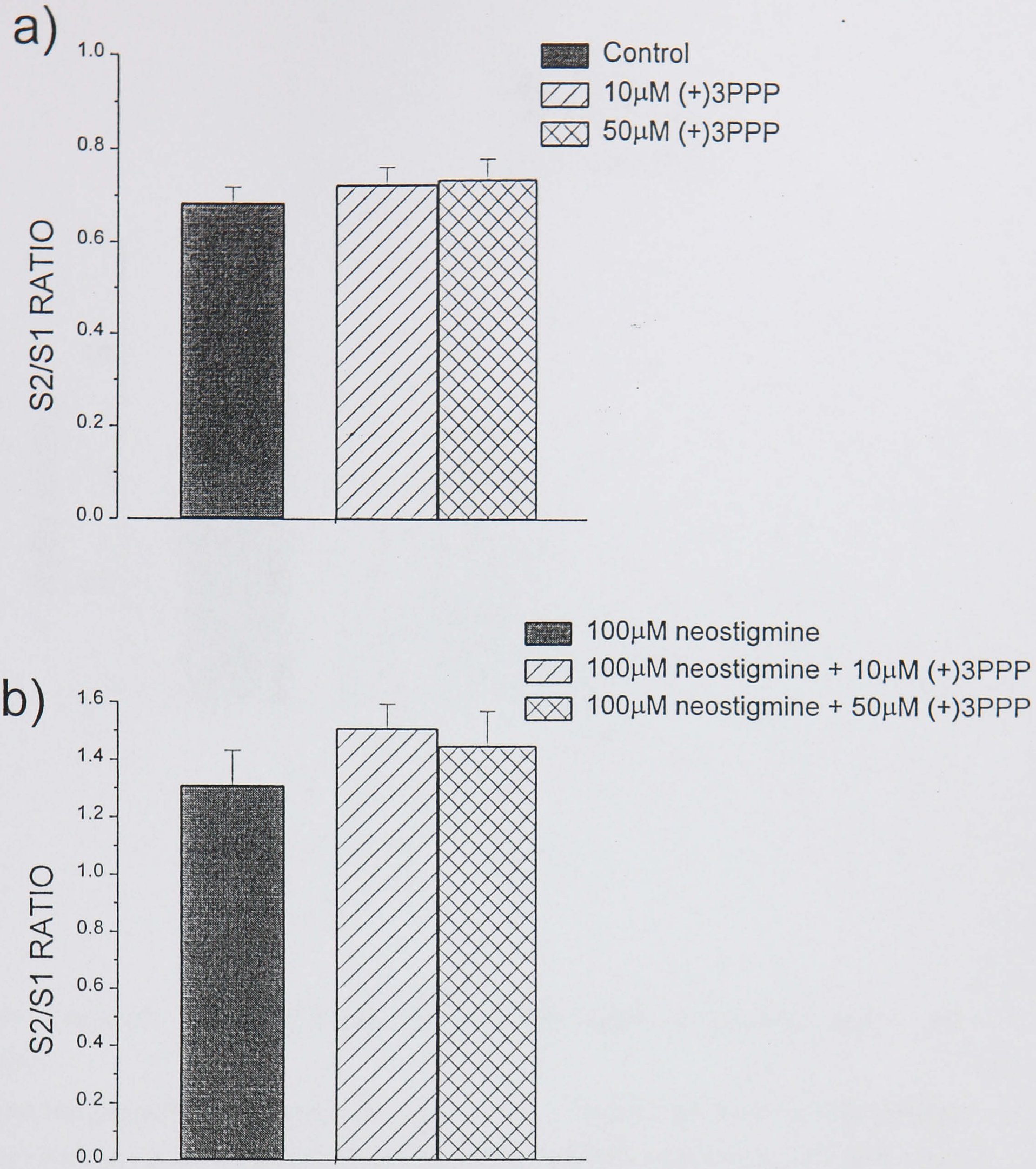


Figure 4.16 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of (+)3-PPP.

a) (+)3-PPP; 10 and 50μM concentrations

b) (+)3-PPP in combination with 100μM neostigmine

Each column represents the S2/S1 ratio as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) observed from four experiments; see Results for details.

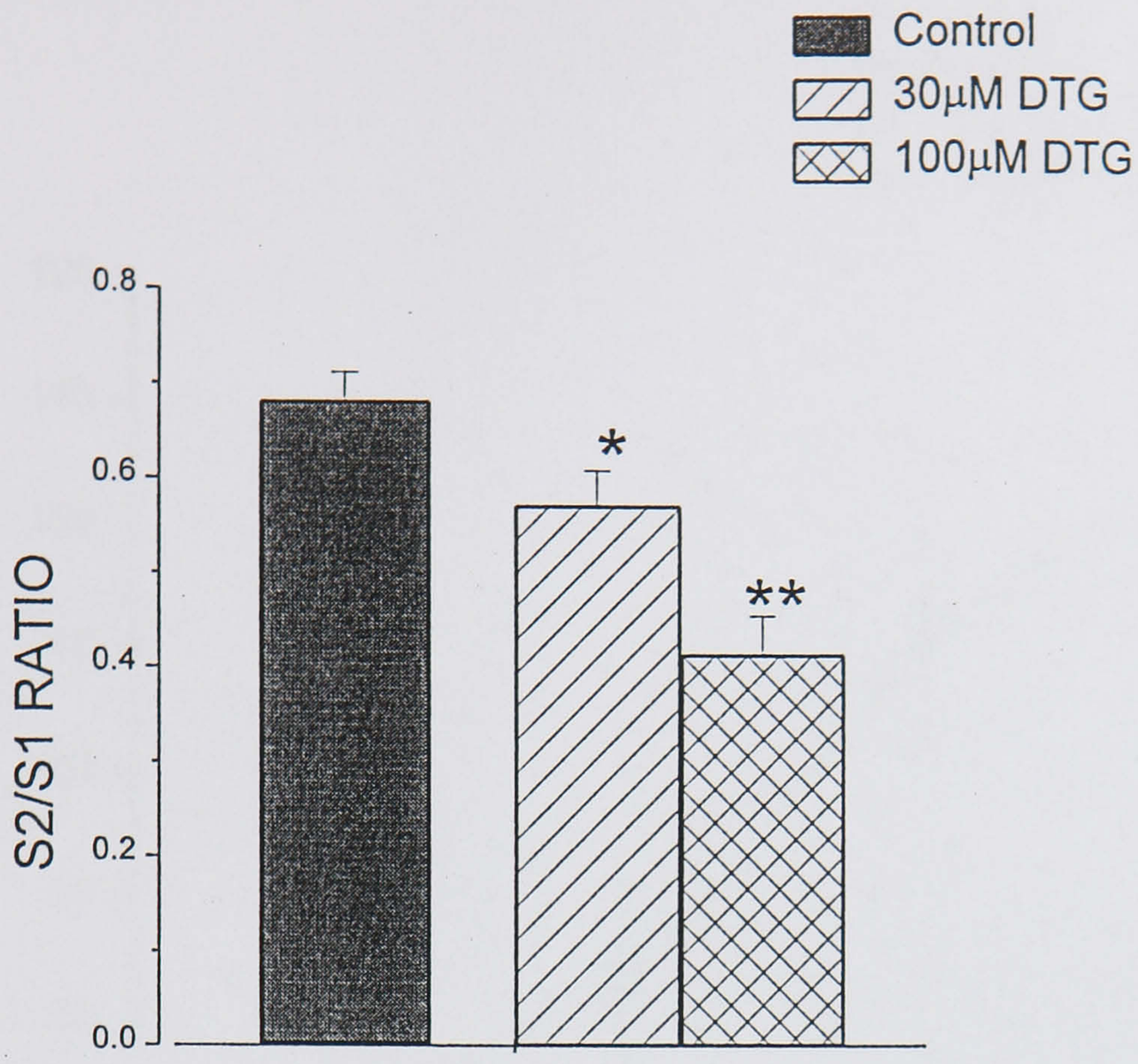


Figure 4.17 The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of DTG.

Each column represents the S2/S1 ratio as the mean ± SEM of the total number of replicate samples (n) observed from four experiments; see Results for details. Significant differences in the S2/S1 ratio compared to the control group are denoted by * $P<0.05$ and ** $P<0.001$.

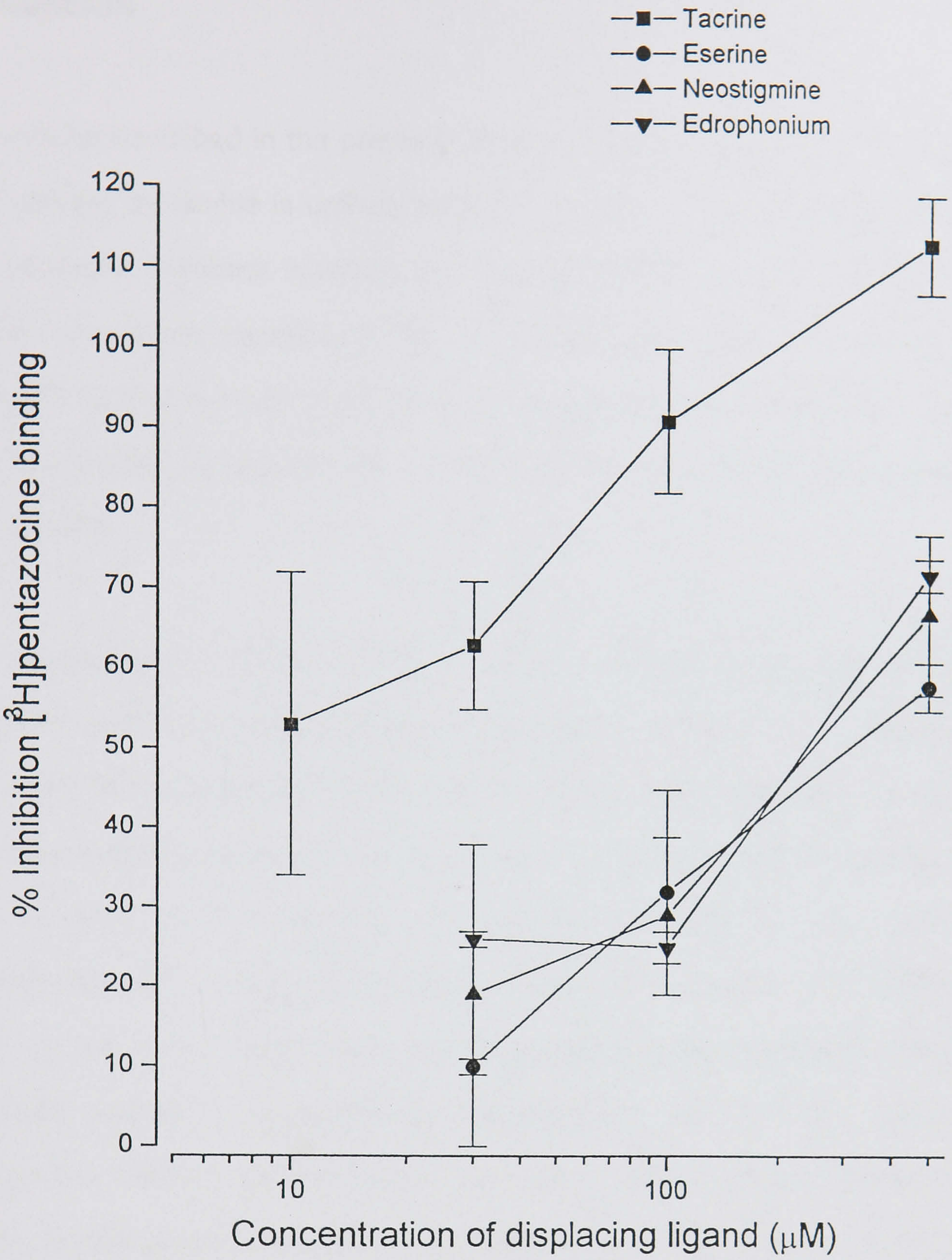


Figure 4.18 Displacement of [^{3}H](+)pentazocine binding in rat hippocampal membrane preparations by the ChE-inhibitors tacrine, eserine, neostigmine and edrophonium .

Each point represents the % inhibition as the mean ± SEM obtained from 3 experiments, each done in triplicate.

DISCUSSION

The results described in the previous chapter led to the conclusion that inhibition of ACh release by tacrine is unlikely to be the result of muscarinic receptor activation. As discussed, feedback inhibition by extracellular ACh seemed unlikely, although a role for muscarinic receptors in the potentiation of release, common to the ChE-inhibitors studied remains to be assessed (Chapter 5). Amongst other possibilities it had been previously reported that tacrine and the other ChE-inhibitors exhibit binding at PCP sites.

The displacement of [^{3}H]TCP binding by tacrine as well as the other cholinesterase inhibitors eserine, neostigmine and edrophonium, (Albin *et al.,* 1988) led to the possibility that an interaction with the PCP site might be responsible for the [^{3}H]ACh release-enhancing effect. Albin *et al.*, (1988) reported that tacrine displaces [^{3}H]TCP with an IC_{50} of 26μM, eserine and edrophonium with an IC_{50} of 100μM and neostigmine with an IC_{50} greater than 100μM. Interaction of ChE-inhibitors at the PCP site was further supported by the observation that tacrine and the cholinesterase inhibitors eserine, neostigmine and edrophonium also provide neuroprotection against the NMDA-mediated toxicity in murine cortical cultures (Davenport *et al.,* 1988). Furthermore in both studies tacrine was shown to be far more effective than the other cholinesterase inhibitors, as was the case in the present [^{3}H]ACh-release experiments, in which the maximum effect of tacrine observed at 30μM concentrations, was comparable to the maximum effect of eserine, edrophonium and neostigmine at 100μM concentrations (Chapter 3).

[^{3}H]TCP, an analogue of PCP, and MK-801 both bind to the cation channel of the NMDA receptor channel complex in the rat brain and have been shown to share many common binding properties (Fagg, 1987). The effects were therefore investigated of these noncompetitive NMDA receptor antagonists MK-801 and PCP,

on the K^+-evoked release of [^{3}H]ACh. MK-801 at concentrations up to 50μM had no effect on evoked-[^{3}H]ACh release from the hippocampal prism preparation (Figure 4.1a) and as Figure 4.1b shows, 100μM concentrations, also had no effect on the neostigmine-induced potentiation of [^{3}H]ACh release.

PCP however increased [^{3}H]ACh release significantly ($P<0.05$), at a concentration of 100μM (Figure 4.2). This is in agreement with the observation of Jackisch *et al.* (1986), that electrically-stimulated release of [^{3}H]ACh from rabbit hippocampal slices was significantly increased by PCP (concentrations not reported in this abstract publication). Junien *et al.*, (1991) however were unable to show an increase in [^{3}H]ACh release from hippocampal slices with PCP although the maximum concentration tested in this latter study was only 30μM.

It may therefore be concluded from these results that the effect of PCP on [^{3}H]ACh release is rather unlikely to be an action mediated via the PCP site inasmuch as MK-801 did not give a similar effect. However PCP is also known to have appreciable σ-binding compared to that of MK-801 (Walker *et al.*, 1990). The characteristics and functional role of PCP and σ sites in the brain have been under extensive investigation for several years. Ligand binding studies and behavioural studies have shown the effects of PCP ligands to overlap with those of σ ligands (Mendelsohn *et al.*, 1985; Mc Cann *et al.*, 1989), although other studies have provided evidence for the existence of distinct PCP and σ sites (Largent *et al.*, 1984a). These studies together with the preliminary results described above (Figures 4.1a & b & 4.2) prompted an investigation of the action of σ ligands on [^{3}H]ACh release from hippocampal prisms. As described in the Introduction to this chapter, the existence of subtypes of σ binding sites (σ1 and σ2) has been based primarily on the stereoselectivity of the σ benzomorphans. For this reason the effect of σ1–selective (+)benzomorphans was investigated in comparison with other non selective, non-

benzomorphan ligands such as haloperidol, (+)3PPP and DTG, which do not discriminate between σ1 and σ2 binding sites.

(+)Benzomorphan σ-ligands

Although (+)NANM has been referred to in the literature as the "prototypic σ ligand", it is also known to label PCP sites (Mendelsohn *et al.,* 1985) and is therefore not strictly selective for σ binding sites. (+)Cyclazocine also has an affinity for the σ and PCP binding site comparable to that of (+)NANM (Mendelsohn *et al.,* 1985; Walker *et al.,* 1990). (+)Pentazocine however, appears to have a greater affinity for σ binding sites as judged by its displacement of [^{3}H](+)3-PPP and [^{3}H]DTG (Largent *et al.,* 1984a; Walker *et al.,* 1990) and a negligible affinity for the PCP site (Largent *et al.,* 1984a). The effects were therefore investigated of these benzomorphan σ ligands (±)pentazocine, (+)cyclazocine and (+)NANM on K^+-evoked [^{3}H]ACh release.

(±)Pentazocine (10 and 50μM) was shown to have no effect on K^+-evoked [^{3}H]ACh release (Figure 4.3) although at 50μM concentrations it was shown to attenuate the potentiation of the evoked release of [^{3}H]ACh caused by the ChE inhibitors tacrine, eserine, neostigmine and edrophonium (Figures 4.4 & 4.5). Interestingly however the S2/S1 ratio was not reduced to control values except in the case of eserine (Figure 4.4b). It is suggested at this stage, that the residual increase over and above control S2/S1 values is likely to be a reflection of the [^{3}H]ACh accumulated as a result of AChE inhibition and equivalent to the HC-3 effect described in Chapter 3. However this is reconsidered in Chapter 6.

(+)Cyclazocine was shown to have a similar effect to that of (±)pentazocine, at concentrations greater than 10μM (Figures 4.7 & 4.8). It is evident from the results shown in Figure 4.8a that maximium inhibition occurs at 30μM concentrations, as 50μM concentrations caused no further inhibition. Furthermore the S2/S1 ratio for

potentiation of release by neostigmine remained significantly greater than the control S2/S1 ratio in the presence of both 30 and 50μM concentrations of (+)cyclazocine. (+)Cyclazocine alone however, had no effect on evoked release at 10μM and 30μM concentrations although at 50μM concentrations it caused a significant reduction of 22% ($P<0.05$, Figure 4.6). Despite this inhibition at higher concentrations, it seems clear that (+)cyclazocine, attenuates the potentiation of release caused by the ChE-inhibitors at a concentration (30μM) which alone, has no effect on K^+-evoked release (see Chapter 6).

In contrast to the above findings with (±)pentazocine and (+)cyclazocine, (+)NANM the "prototypic σ ligand" has been reported to increase the release of ACh in the CNS *in vivo* and *in vitro*. *In vivo*, microdialysis has demonstrated that (+)NANM increases extracellular ACh levels in the presence of eserine in rat frontal cortex (Matsuno *et al.,* 1992). (+)NANM also enhances electrically evoked [^{3}H]ACh release in rabbit hippocampal slices (Jackisch *et al.,* 1987) as well as K^+-evoked release in cortical and thalamic slices, in the presence of eserine or carbachol (Siniscalchi *et al.,* 1987). Moreover Junien *et al.*, (1991) reported that (+)NANM or JO1784, a novel σ ligand, increased K^+-evoked release of [^{3}H]ACh from rat hippocampal slices in the presence of HC-3 (10μM).

However in the present experiments (Figure 4.9), (+)NANM had no effect on K^+-evoked release even though HC-3 was added to conform to the procedure of Junien *et al.,* (1991). Neither did it affect the neostigmine-induced potentiation of [^{3}H]ACh release as shown in Figure 4.10a. However the levorotatory (-) isomer of NANM has been previously shown to have κ opioid agonist properties (Quirion *et al.,* 1987) and opioid receptors are known to be involved in the inhibition of ACh release in the brain (Lapchak *et al.,* 1988). The possibility was considered therefore, that in the present experiments, potentiation of [^{3}H]ACh release by the σ activity of (+)NANM (as observed in the aforesaid studies) might have been opposed by some opioid activity.

However as Figure 4.10b shows, naloxone did not alter the effect of (+)NANM on K^+-evoked release and neither did it alone affect control K^+-evoked release.

It is not clear therefore why the present results with (+)NANM are at variance with those previously reported, nor is it clear why the effects with (±)pentazocine and (+)cyclazocine (Figures 4.4, 4.5, 4.7 & 4.8) differ from those with (+)NANM (Figure 4.10a). However it has also been previously reported that (+)NANM has a muscarinic antagonist effect which blocks negative feedback inhibition by ACh (Siniscalchi *et al.*, 1987). The role of muscarinic autoreceptors is considered further in the following chapter.

Relevant to the present discussion, the displacement of [^{3}H]QNB, by the three σ ligands was compared (Figure 4.11). (+)NANM was clearly not more effective in the displacement of [^{3}H]QNB than was (+)cyclazocine, and indeed (±)pentazocine, was markedly more effective than both (+)NANM and (+)cyclazocine. (+)Pentazocine and (+)NANM have both been shown to be muscarinic antagonists as is evident by their ability to inhibit carbachol-induced phosphoinositide turnover (IC_{50} 32 & 213μM respectively) (Candura *et al.*, 1990). They also inhibit carbachol-induced contractions in the guinea pig ileum preparations and oxotremorine-induced analgesia in mice (Vargas & Pechnick, 1991). It may seem unlikely therefore, that muscarinic receptor antagonism would explain the distinction observed between (+)NANM on the one hand and (+)cyclazocine and (±)pentazocine, on the other, in the present experiments. This is reconsidered in Chapters 5 and 6.

It might be tentatively concluded at this stage that potentiation of [^{3}H]ACh release by the ChE-inhibitors involves their action at σ receptors and that this is attenuated by (+)cyclazocine and (±)pentazocine. The fact that PCP itself causes potentiation (Figure 4.2) may be thought to support this idea although it is known to have other

properties (see next chapter). The results discussed and presented so far however, fail to explain the lack of effect of (+)NANM on K^+-evoked release.

Non-benzomorphan σ ligands

One of the pharmacological characteristics of the σ binding site is its high affinity for haloperidol, a non-benzomorphan, which has been suggested to act as an antagonist at this site. Recently Junien *et al.* (1991) demonstrated that the potentiation of K^+-evoked [^{3}H]ACh release from rat hippocampal slices by (+)NANM or JO 1784 was antagonized by haloperidol (0.3μM). This compound was therefore tested in the present series of experiments; on the potentiation of [^{3}H]ACh release caused by the ChE-inhibitors and also on of K^+-evoked release of [^{3}H]ACh. Haloperidol at a concentration of 1μM was ineffective (results not shown), however at 30μM concentrations, the potentiation caused by each of the four ChE-inhibitors was abolished - i.e the S2/S1 ratios were reverted to control values (Figure 4.12, Table 4.8). This effect appeared to be a functional antagonism since haloperidol alone, also inhibited K^+-evoked release by 33% ($P<0.001$) and 53% ($P<0.001$) at concentrations of 10 and 30μM respectively, while at 100μM haloperidol, the S2 stimulation was completely abolished (Figures 4.13 & 4.14, Table 4.7).

In addition to its high affinity for σ binding sites, haloperidol has also been shown to bind with high affinity to receptors for dopamine, noradrenaline (α1), 5HT and very weakly to muscarinic binding sites (cited in Monnet *et al.,* 1991a) while showing no affinity for the PCP sites (Largent et al., 1984a; Quirion *et al.,* 1987). In order to determine whether the inhibition of [^{3}H]ACh release caused by haloperidol was due to dopamine/ACh interactions, the D1 and D2 antagonists SCH 23390 and sulpiride were tested and found to have no effect on the neostigmine-induced potentiation of [^{3}H]ACh release (Figures 4.15b & 4.15c). Furthermore sulpiride, which has minimal

affinity for the σ site (Rao *et al.*, 1991) was also shown to have no effect on the K^+-evoked release of [^{3}H]ACh (Figure 4.15a).

The effects of the non-benzomorphan, ligands (+)3-PPP and DTG were also investigated (Figures 4.16 & 4.17). These σ ligands display a high affinity for the σ binding site and are frequently used in ligand binding studies, although DTG does not differentiate between the subtypes. (+)3-PPP which is known to have a slightly higher affinity for the σ1 binding sites (K_D ~ 5 and 440nM for σ1 and σ2 respectively)(Walker *et al.*, 1990), had no effect on [^{3}H]ACh release either K^+-evoked or neostigmine-potentiated (Figures 4.16a & 4.16b). DTG however, was shown to inhibit K^+-evoked [^{3}H]ACh release in a concentration-dependent manner similar to haloperidol as shown in Figures 4.14 & 4.17. Concentrations of 30 and 100μM DTG gave inhibitions of 16% ($P<0.05$) and 39% ($P<0.001$) respectively (Figure 4.17). Similar effects in the rat hippocampus have also been also reported by Junien *et al.*, (1991).

DTG and its congeners have also been shown to have similar effects in the periphery, where they are reported to inhibit [^{3}H]ACh release and also 5-HT-induced and electrically-induced contractions in isolated guinea pig ileum (Campbell *et al.*, 1989). The potency of these ligands in inhibiting guinea-pig ileum contractions was shown to be highly correlated with their binding affinity to the σ binding sites. Although DTG has been shown to have weak muscarinic antagonist activity (Hudkins & De Haven-Hudkins, 1991; Vargas & Pechnick, 1991), σ ligands lacking affinity to the muscarinic receptors were also found to inhibit the contractions as well as [^{3}H]ACh release from this preparation. Furthermore DTG congeners structurally related to DTG but lacking affinity for the σ binding sites were found not to alter [^{3}H]ACh release. The inhibition of [^{3}H]ACh release by these ligands was therefore ascribed to an action at the σ binding sites (Campbell *et al.*, 1989).

The observation that tacrine (Chapter 3) and the non-benzomorphan ligands haloperidol and DTG, strongly inhibit K^+-evoked [^{3}H]ACh release may be of significance. It is possible that the tacrine-induced inhibition of [^{3}H]ACh release is similarly mediated by σ binding sites. Thus the overall picture emerging appears to involve σ activity in both the potentiation of [^{3}H]ACh release by ChE-inhibitors and paradoxically, also in the inhibition of K^+-evoked release.

Furthermore it is interesting to observe that the ChE-inhibitors tacrine, eserine, neostigmine and edrophonium, as shown in Figure 4.18, displace the σ1-selective radioligand [^{3}H](+)pentazocine from hippocampal membrane preparations. Nevertheless although tacrine displaced [^{3}H](+)pentazocine with a IC_{50} of approximately 10μM, eserine, neostigmine and edrophonium, showed considerably weaker affinity for the σ binding sites with IC_{50} values greater than 100μM. The results from these radioligand binding studies therefore make it difficult to suggest that the potentiation of K^+-evoked [^{3}H]ACh release by the ChE-inhibitors eserine, neostigmine and edrophonium is due to an interaction with σ binding sites, although it is clear from the results presented in this chapter, that this potentiation is attenuated by the σ ligands (±)pentazocine and (+)cyclazocine.

Since the ligands (+)NANM, (+)cyclazocine and (±)pentazocine which are known to be predominantly σ1-selective, have no effect on K^+-evoked [^{3}H]ACh release in the present studies, it is tempting to speculate that the inhibition of [^{3}H]ACh release observed with the non-benzomorphan, non-selective σ ligands haloperidol and DTG is due to an action at the σ2 binding sites. It is therefore suggested that the inhibition of [^{3}H]ACh release observed with concentrations of 100 and 200μM tacrine, might also be mediated by a mechanism similar to that responsible for the inhibition of [^{3}H]ACh release by haloperidol and DTG. However it is noted that the concentrations of haloperidol and DTG required to produce inhibition are in the micromolar range

compared to the nanomolar IC_{50}s observed in radioligand binding studies (see Chapter 6).

In addition to its actions at the σ and muscarinic binding sites, the σ benzomorphans cyclazocine, NANM and pentazocine have also been shown to act non competitively at the nicotinic receptors. King and Aronstam (1983), showed that the above mentioned benzomorphans inhibited the binding of [^{3}H]perhydrohistrionicotoxin ([^{3}H]H_{12}-HTX) to ion channel sites in torpedo ACh receptor complexes with IC_{50} values of approximately 5μM. Interestingly, previous studies by various workers have also shown that the ChE-inhibitors tacrine, eserine and neostigmine also interact with nicotinic receptors (De Sarno *et al.,* 1989; Fiekers, 1985; Nilsson *et al.,* 1987; Shaw *et al.,* 1985; Sherby *et al.,* 1985). It is possible therefore that the potentiation of K^+-evoked [^{3}H]ACh release is due to an interaction at the nicotinic receptor and this will therefore be further investigated in the following chapter.

CHAPTER 5

Modulation of [^{3}H]ACh release by actions at muscarinic and nicotinic autoreceptors

INTRODUCTION

It was observed from the results described in Chapter 4 that the benzomorphan σ ligands (+)cyclazocine and (±)pentazocine blocked the potentiation of [^{3}H]ACh release caused by the ChE-inhibitors tacrine, eserine, neostigmine and edrophonium. However the binding affinities of these ChE-inhibitors for the σ binding sites labelled with [^{3}H](+)pentazocine were unimpressive (Figure 4.18). Thus it was concluded that the potentiation of [^{3}H]ACh release by the ChE-inhibitors was unlikely to be due to an interaction with the σ binding sites. However as mentioned previously, these benzomorphan σ ligands as well as (+)NANM have all been shown to act noncompetitively at the nicotinic receptors. The binding of [^{3}H]perhydrohistrionicotoxin to nicotinic ion channel sites was inhibited by these benzomorphans with IC_{50} values of approximately 5μM (King & Aronstam, 1983). Furthermore several workers have also shown that the ChE-inhibitors themselves interact with nicotinic receptors. Here in this chapter, the role of nicotinic and muscarinic receptors in the modulation of [^{3}H]ACh release is considered.

One of the mechanisms regulating ACh release in the CNS is by presynaptic auto and heteroreceptors. A great deal of interest has been focused on the characterization and pharmacological manipulation of these presynaptic autoreceptors by cholinergic agents, which may possibly improve cholinergic transmission if these receptors differ pharmacologically from the postsynaptic ones.

It is now well documented, that ACh release from cholinergic axon terminals in the CNS and in the periphery are under presynaptic feedback control through muscarinic autoreceptors. Autoreceptor-mediated feedback, a process controlling the synaptic release of transmitters was first described by Langer (1977) wherein endogenous ligands inhibited their own release by activating presynaptic "autoreceptors". The inhibition of depolarization-dependent ACh release by ACh itself was however,

observed long before (Mitchell, 1963; Szerb, 1964; Polak and Meeuws, 1966) and a large body of evidence has since been documented supporting the autoreceptor mediated regulation of ACh release in the periphery and CNS. Muscarinic ACh autoreceptors mediating inhibition of ACh release from central cholinergic neurons have been investigated in rat cortical brain slices and synaptosomes (Hadhazy and Szerb,1977; Rospars *et al.,*1977; Raiteri *et al.,*1984; Meyer and Otero,1985) and also in rat hippocampal nerve endings (Nordstrom and Bartfai, 1980; Marchi *et al.,*1981; Raiteri *et al.,*1984). It is commonly observed that muscarinic agonists decrease ACh release and a property common to these ACh release-inhibiting autoreceptors is their sensitivity to atropine.

ACh release has also been shown to be modulated by heteroreceptors such as those for 5-HT, noradrenaline, dopamine, adenosine and opioid receptors. However in each brain area the cholinergic nerve endings are equipped with different sets of presynaptic receptors making possible a differential regional modulation. For instance Marchi *et al.,* 1983, demonstrated that the activation of striatal muscarinic autoreceptors is less effective in reducing ACh release in the striatum than in the cortex and hippocampus. Similarly adenosine inhibition of ACh release is more effective in the hippocampus than in the cerebral cortex (Pedata *et al.,* 1986). Furthermore the heteroreceptors modulating ACh release have also been shown to vary in subtype in the different regions of the brain. For example, 5-HT heteroreceptors mediating the inhibition of ACh release in the hippocampus have been shown to belong to the 5-HT_{1B} subtype in the hippocampus (Maura & Raiteri, 1986), 5-HT_3 in the cortex (Barnes *et al.,* 1989) and 5-HT_2 in the striatum (cited in Maura & Raiteri, 1986). The inhibition of ACh release by NA involving α_2 heteroreceptors has been reported only in the cortex (Vizi, 1980; Beani *et al.,* 1986) as far as is known and likewise, the potentiation of evoked DA release by muscarinic heteroreceptors is best known in the striatum (Schoffelmeer *et al.,* 1986; Ono *et al.,* 1988; Raiteri *et al.,* 1984) and the cortex (Marchi & Raiteri, 1985a).

There have been many studies attempting to correlate the heterogeneity of muscarinic receptors with neurochemical function in the CNS. In many studies, muscarinic receptors subtypes in the brain have been investigated by using neurotransmitter release as a functional parameter and by comparing the effects of agonists and antagonists. Recent studies dealing with the ACh release-inhibiting autoreceptors have led to their characterization and the evidence suggests that they are of the M2 muscarinic receptor subclass in the striatum (Weiler, 1989; James & Cubeddu, 1987; Schoffelmeer *et al.,* 1986), cortex (Marchi & Raiteri, 1985a) and hippocampus (Marchi & Raiteri, 1985a; Pohorecki *et al.,* 1988; Ono *et al.,* 1988). Furthermore there is increasing evidence that muscarinic receptors are located also on dopaminergic nerve terminals (Raiteri *et al.,* 1982; Schoffelmeer *et al.,* 1986) and it has been shown that the activation of these heteroreceptors which potentiate evoked release of DA in the cerebral cortex (Marchi & Raiteri, 1985a; Marchi & Raiteri, 1985b) and striatum (Raiteri *et al.,* 1984; Schoffelmeer *et al.,* 1986; Ono *et al.,* 1988), belong to the M1 subtype.

Evidence supporting the existence of nicotinic receptors on cholinergic nerve endings (Yoshida & Imura, 1979; Romano & Goldstein, 1980; Schwartz *et al.*, 1982) as well the ability of nicotine to promote transmitter release led to the idea and the possibility of a direct presynaptic action of nicotine. Nicotine has been shown to evoke release of neurotransmitter and also to potentiate release evoked by nerve impulses or by other depolarizing stimuli such as high K^+ concentrations (Westfall, 1974; Beani *et al.,* 1985).

It was found that nicotinic receptor stimulation enhances the release of ACh (Beani *et al.,* 1989; Chiou *et al.,* 1970; Wilkie *et al.,* 1993) and aspartate (Beani *et al.,* 1989) from the cortex, NA and DA from the hypothalamus and the striatum (Goodman, 1974; Sandor *et al.,* 1991). It appears that the best characterized presynaptic action of nicotine concerns the enhancement of ACh release from the hippocampus (Beani

et al., 1989; Wilkie *et al.,* 1993; Araujo *et al.,* 1988; Moss & Wonnacott, 1985; Wonnacott *et al.*, 1987) and DA release from the striatum. The nicotinic facilitation of DA release *in vivo* from striatal nerve terminals has been previously reported (cited in Marchi *et al.,* 1983), as has DA release from slice (Giorguieff *et al.,* 1976; Goodman 1974) and synaptosomal preparations (Clarke *et al.,* 1994; Giorguieff-Chesselet *et al.,* 1979; Rapier *et al.,* 1984; Wonnacott, 1991). Nicotine-evoked release of [^{3}H]ACh from hippocampal synaptosomes has also been well documented by Wonnacott *et al.,* (Moss & Wonnacott, 1985; Wonnacott *et al.,* 1989; Wilkie *et al.,* 1993). Furthermore, nicotine-evoked [^{3}H]DA release from rat striatal synaptosomes has been shown to be blocked by the ChE-inhibitors tacrine, eserine and neostigmine (Clarke *et al.,* 1994).

It has been suggested by Wonnacott *et al.,* (1989) that these striatal presynaptic nicotinic receptors are similar to those found on hippocampal synaptosomes, which are involved in the facilitation of ACh release. It is reported that these striatal and hippocampal presynaptic receptors (which modulate DA and ACh release) are blocked by DHβE, but are insensitive to α-bungaratoxin (Wonnacott *et al.,* 1989; Wonnacott, 1991). Araujo *et al.,* (1988) have also shown that the nicotinic agonist methylcarbamylcholine (MCC) enhanced [^{3}H]ACh release from rat hippocampal and cortical slices but not from striatal slices. This effect of MCC to increase ACh release was found to be antagonized by the nicotinic antagonists DHβE and *d*-tubocurarine but not by α-bungarotoxin .

The hypothesis that nicotinic receptors increase the release of ACh (at the neuromuscular junction) was first suggested by Beani *et al.,* in 1964 and has since been reviewed and demonstrated *in vivo* (Armitage *et al.,* 1969; Erickson *et al.,* 1973; Beani *et al.,* 1989) and *in vitro* (Beani *et al.,* 1989; Rowell & Winkler, 1984; Beani *et al.,* 1985; Moss & Wonnacott, 1985; Wilkie *et al.,* 1993; Meyer *et al.,* 1987; Araujo *et al.,* 1988; Loiacono & Mitchelson, 1990) in the CNS and the periphery

(Wessler *et al.*, 1986; Bowman *et al.*, 1988). Beani *et al.* (1985) showed that nicotine as well as the nicotinic agonist cytisine facilitated the electrically stimulated release of [^{3}H]ACh from cortical slices of the guinea pig brain and was blocked by *d*-tubocurarine. In the periphery also, Wessler *et al.*, (1986) was able to demonstrate a positive feedback mechanism mediated by presynaptic nicotinic receptors on the motor nerve terminals (rat phrenic nerve). They showed that the nicotinic agonist DMPP (dimethylphenylpiperazinium) enhanced the electrically stimulated release of [^{3}H]ACh in a concentration-dependent manner which was antagonized by *d*-tubocurarine.

Evidence for the existence of several subtypes of nicotinic receptors has been accumulated from pharmacological, electrophysiological and immunohistochemical studies. Differences in single channel conductances and sensitivity to snake neurotoxins such as α bungarotoxin and neuronal bungarotoxin suggest that there are physiological, pharmacological and structural differences between nicotinic receptors across species, tissues and even within tissues. Immunohistochemical and immunochemical analyses also support nicotinic receptor heterogeneity and are beginning to reveal relationships between nicotinic receptor-like antigens, specific radioligand binding sites and specific nicotinic receptor subunits (Lukas & Bencherif, 1992). The application of recombinant DNA techniques in the characterization and cloning of nicotinic receptor subunit-encoding genes and mRNA have been of significant importance in the understanding of the diversity of nicotinic receptors.

It has also been reported by many workers that neostigmine, eserine and other ChE inhibitors, at higher concentrations have various effects that are unrelated to AChE inhibition. These include:

- an interaction with nicotinic ACh receptor; the binding of neostigmine and eserine to these receptors has been demonstrated by their competitve inhibition of specific [^{3}H]ACh binding in the electric organ of the Torpedo and the

competitive inhibition of the ^{125}I-α-bungarotoxin binding to the aplysia receptors (Sherby *et al.*, 1985). Binding studies have also revealed these ChE inhibitors to interact directly with the nicotinic receptor channel in different ways also causing desensitization (which is measured as increased potency of the agonist to inhibit binding of the radioligand e.g ^{125}I-α-bungarotoxin to the receptor sites) (Sherby et al., 1985; Bloch & Stallcup, 1979). Furthermore eserine has been shown to be a potent blocker of the receptor's activated channel conformation (Sherby et al., 1985).

- an agonist as well as an antagonist effect on the nicotinic ACh receptor: Electrophysiological studies have demonstrated that neostigmine and eserine have agonist activity similar to ACh (Shaw et al., 1985; Harvey & Dryden, 1974) and carbachol (Bloch & Stallcup, 1979). However neostigmine is also known to block ACh receptors in the voltage clamped twitch fibres of the costocutaneous muscles of the garter snake (Fiekers, 1985). Furthermore neostigmine, eserine and tacrine have been shown to produce a concentration-dependent blockade of nicotine-evoked [^{3}H]DA from rat striatal synaptosomes (Clarke *et al.*, 1994)
- direct and indirect presynaptic effects affecting the release of ACh; It was found by Chang and Hong (1986) using intracellular recording techniques with glass microelectrodes, that train stimulation (50-200 Hz) of the phrenic nerve of intact or cut mouse diaphragm induced an accumulative depolarisation of the end plate and triggered after a few pulses, an "all or none" regenerative depolarisation when AChE was inhibited by neostigmine. It was suggested that the ACh accumulated in the synaptic cleft acts on the nerve terminal cholinoceptor causing a depolarisation which, at a threshold depolarisation produced a regenerating release of ACh. *D*-Tubocurarine was shown to block the regenerating depolarisation (Chang & Hong, 1986).

The ChE-inhibitors tacrine, eserine, neostigmine and edrophonium which are also known to have muscarinic and nicotinic properties, have been shown in the previous

chapters to potentiate the K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms. It has also been observed that the ChE-inhibitors tacrine and eserine enhanced evoked-[^{3}H]ACh release from postmortem Alzheimer brain tissue in a manner which was blocked by *d*-tubocurarine and DHβE (Nordberg *et al.*, 1989). Facilitation of synaptic transmission by muscarinic and nicotinic agents therefore, seems a rational approach and has been considered as a possible therapeutic approach for the treatment of Alzheimer's disease. The modulation of hippocampal ACh release by the interaction of cholinergic agents with muscarinic and nicotinic receptors and the possibilty of the ChE-inhibitors having a direct action on cholinergic transmission via these receptors, was therefore considered in this Chapter.

METHODS

1. Release experiments

Preparation of tissue

Hippocampal prisms were prepared, preloaded with [^{3}H]choline and superfused as described in Chapter 2 - General Methods and Materials

MATERIALS

Chemicals

From Semat Technical UK Ltd, St. Albans, Hertfordshire, (Research Biochemicals Incorporated, USA):

DHβE hydrobromide, (+)cyclazocine and (+)NANM hydrochloride

From Sigma Chemical Co. Ltd., Poole, Dorset, UK:

Oxotremorine sesquifumarate salt, carbachol chloride, nicotine salicylate salt, DMPP iodide, mecamylamine hydrochloride, *d*-tubocurarine chloride, hexamethonium bromide and (±)pentazocine hydrochloride

From Burroughs Wellcome & Co., London:

Atropine sulphate

RESULTS

1. The effects of the muscarinic antagonist atropine on:

The potentiation of [^{3}H]ACh release caused by the ChE inhibitors

Atropine at 10μM concentrations produced a significant enhancement of 19, 19, 28 and 16% respectively of the potentiation of [^{3}H]ACh release caused by the ChE inhibitors tacrine (30μM), eserine (100μM), neostigmine (100μM) and edrophonium (100μM), see Table 5.2 and Figure 5.1.

The inhibition of [^{3}H]ACh release caused by tacrine

Tacrine at 100 and 200μM concentrations caused a 24% ($P<0.001$) and 62% ($P<0.0001$) inhibition of [^{3}H]ACh release. At 100μM concentrations of tacrine however, this inhibition was reversed by 10μM atropine. Atropine had no effect on the inhibition of release caused by 200μM tacrine, see Table 5.1 below and Figure 5.2.

CONCENTRATION	S2/S1 RATIO		
TACRINE	CONTROL	TACRINE	TACRINE+ATROPINE
100μM	0.675 ± 0.019 (n=15)	0.516 ± 0.027*(n=15)	0.622 ± 0.025 (n=15)
200μM	0.704 ± 0.012 (N=3)	0.266 ± 0.039**(N=3)	0.247 ± 0.039 (N=3)

TABLE 5.1

The effect of atropine on the tacrine-induced inhibition of K^+-evoked [^{3}H]ACh release. The test samples were superfused with tacrine or tacrine and atropine 10 min prior to and during the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of means (N) or the means ± SEM of the total number of replicate samples (n) observed from 3 to 4 experiments, depending on the analysis of variance; see Methods (Chapter 2) for details. Significant differences compared to the S2/S1 ratio of the ChE-inhibitor alone are denoted by *$P<0.001$ and ** $P<0.0001$.

The oxotremorine-induced inhibition of [^{3}H]ACh release

As shown in Figure 5.3a oxotremorine at 100μM concentrations, caused a 22% inhibition of [^{3}H]ACh release - control S2/S1 0.686 ± 0.054 N=3; 100μM oxotremorine S2/S1 0.534 ± 0.033 N=3 (P<0.001). This inhibition was antagonized by 10μM atropine to S2/S1 values comparable to those of control - 100μM oxotremorine and 10μM atropine S2/S1 0.748 ± 0.024 N=3 (P>0.05).

K^+-evoked release of [^{3}H]ACh

Atropine at 10μM concentrations had no effect on K^+-evoked [^{3}H]ACh release - Figure 5.3b. Control S2/S1 0.705 ± 0.030 n=15; 10μM atropine S2/S1 0.670 ± 0.025 n=16 (P>0.05).

2. The effects of (+)NANM on:

The carbachol-induced inhibition of [^{3}H]ACh release

As shown in Figure 5.4a, (+)NANM at 30μM concentrations was found to antagonize the inhibition of [^{3}H]ACh release caused by carbachol (50μM); control S2/S1 0.639 ± 0.019 n=13; 50μM carbachol S2/S1 0.529 ± 0.032, n=16 (P<0.05); 50μM carbachol and 30μM (+)NANM S2/S1 0.628 ± 0.026, n=14.

The neostigmine-induced potentiation of [^{3}H]ACh release in the presence of atropine

(+)NANM at 30μM concentrations was found to oppose the potentiation of [^{3}H]ACh release caused by neostigmine (100μM) and atropine (10μM) together; 100μM neostigmine and 10μM atropine S2/S1 1.238 ± 0.071, n=16; 100μM neostigmine and 10μM atropine and 30μM (+)NANM S2/S1 1.056 ± 0.035, n=16, (P<0.01), Figure 5.4b.

	CONTROL	CHE-INHIBITOR	CHE-I +10μM ATROPINE
TACRINE (30μM)	0.673 ± 0.027 (n=13)	1.079 ± 0.041 (n=14)	1.282 ± 0.052* (n=13)
ESERINE (100μM)	0.711 ± 0.023 (n=14)	1.133 ± 0.032 (n=15)	1.348 ± 0.045** (n=14)
NEOSTIGMINE (100μM)	0.690 ± 0.027 (n=12)	1.152 ± 0.032 (n=15)	1.478 ± 0.056** (n=14)
EDROPHONIUM (100μM)	0.595 ± 0.029 (n=15)	1.066 ± 0.043 (n=16)	1.239 ± 0.042* (n=15)

TABLE 5.2

The effect of atropine on the ChE-inhibitor-induced potentation of K^+-evoked [^{3}H]ACh release.

The test samples were superfused with ChE-inhibitor or ChE-inhibitor and atropine 10 min prior to and during the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of the total number of replicates (n) observed from four experiments; see Methods (Chapter 2) for details. Significant differences compared to the ChE-inhibitor alone are denoted by *P<0.01 and ** P<0.001.

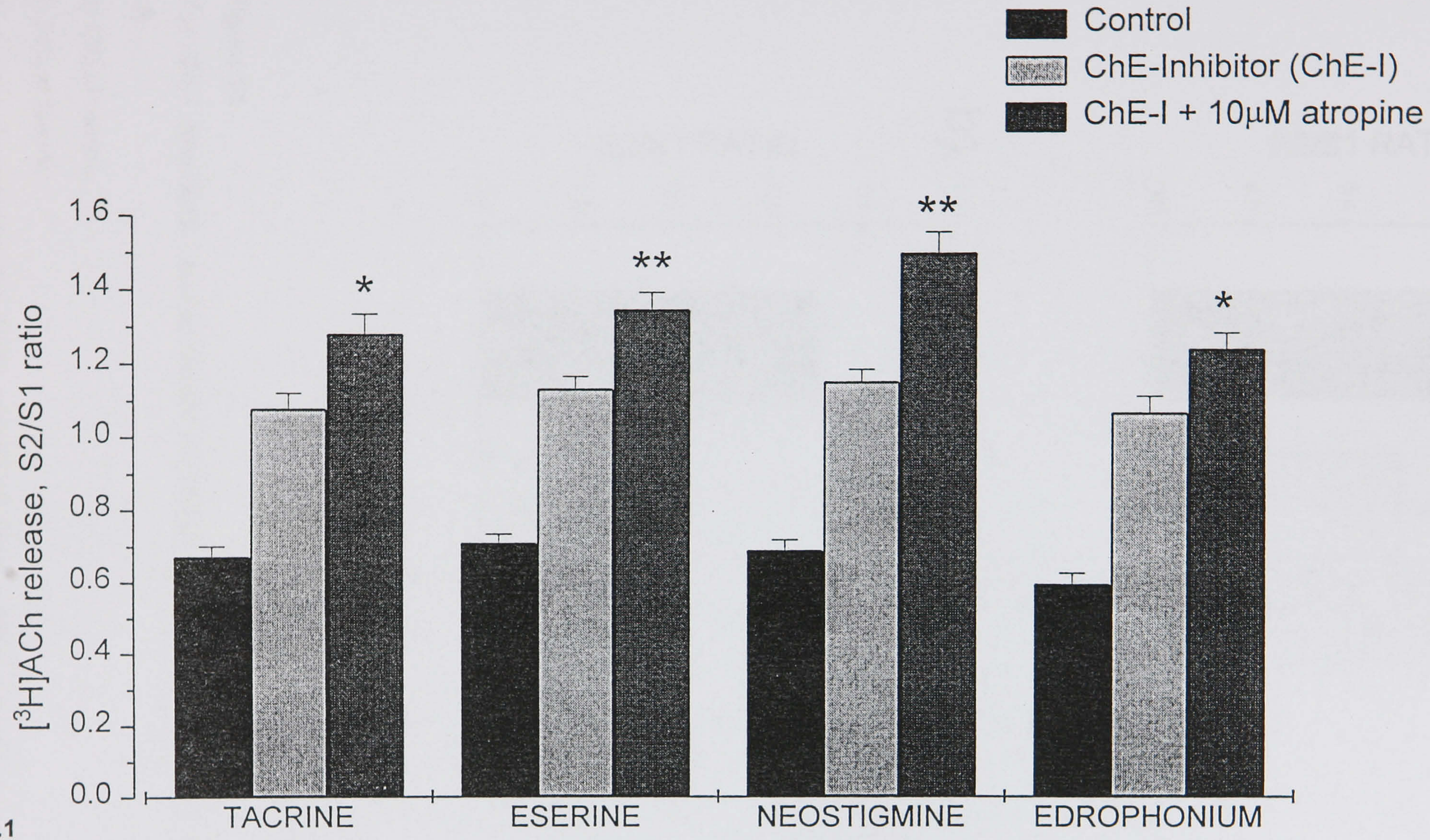


Figure 5.1

The S2/S1 ratios of K^+-evoked release of [^{3}H]ACh release from rat hippocampal prisms - the effect of atropine on the potentiation of [^{3}H]ACh release by the ChE-inhibitors tacrine, eserine, neostigmine and edrophonium.

Each column represents the S2/S1 ratio as the mean ± SEM of the total number of replicate samples (n) obtained from 4 experiments; see Table 5.2 for details.

Significant differences in S2/S1 ratio compared to that of the ChE-inhibitor alone are denoted by * $P<0.01$ and ** $P<0.001$.

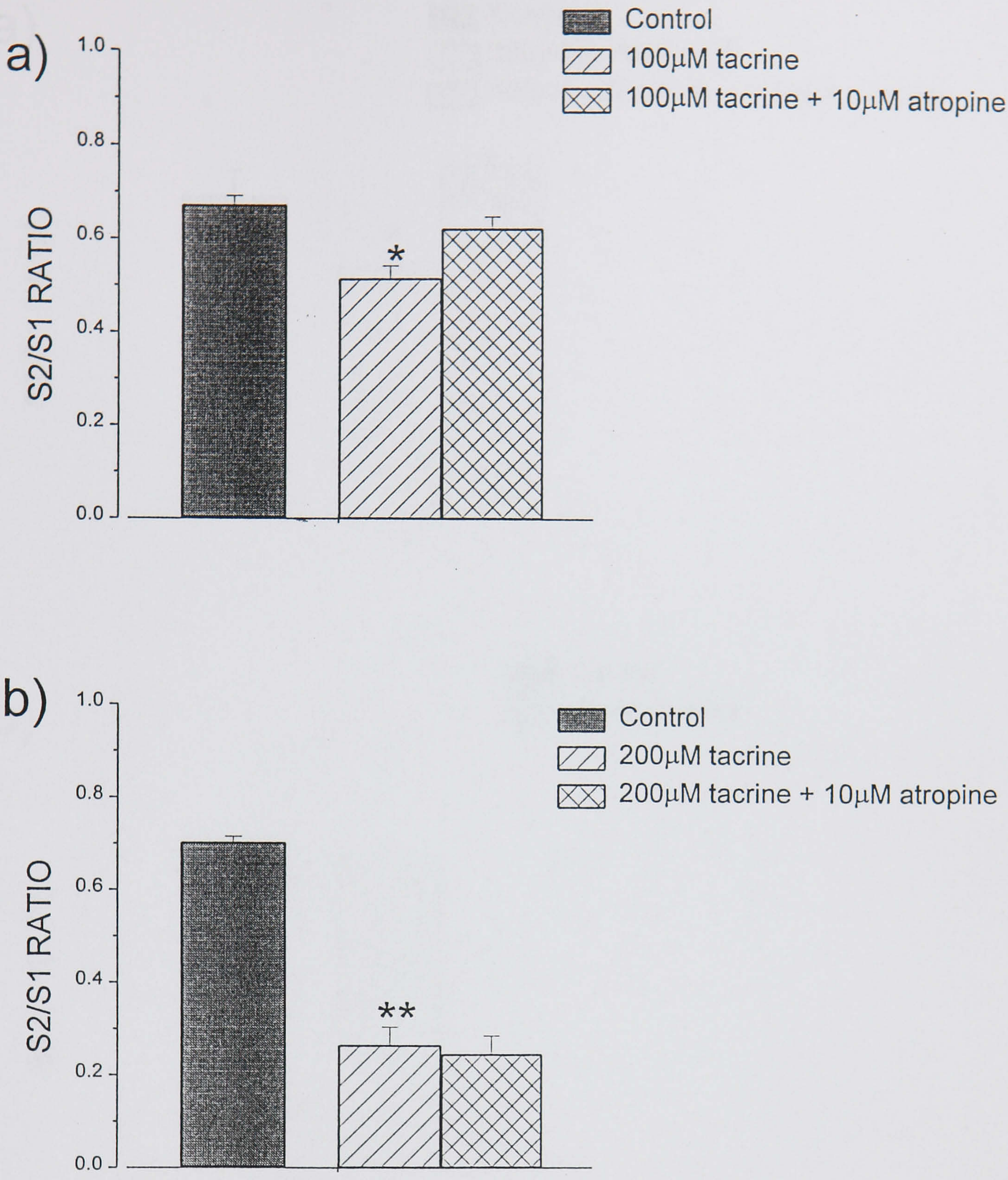


Figure 5.2

The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of atropine on:

a) 100μM tacrine

b) 200μM tacrine

Each column represents the S2/S1 ratio as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) obtained from three to four experiments; see Table 5.1 for details. Significant differences compared to the control S2/S1 ratio are denoted by * $P<0.001$ and ** $P<0.0001$.

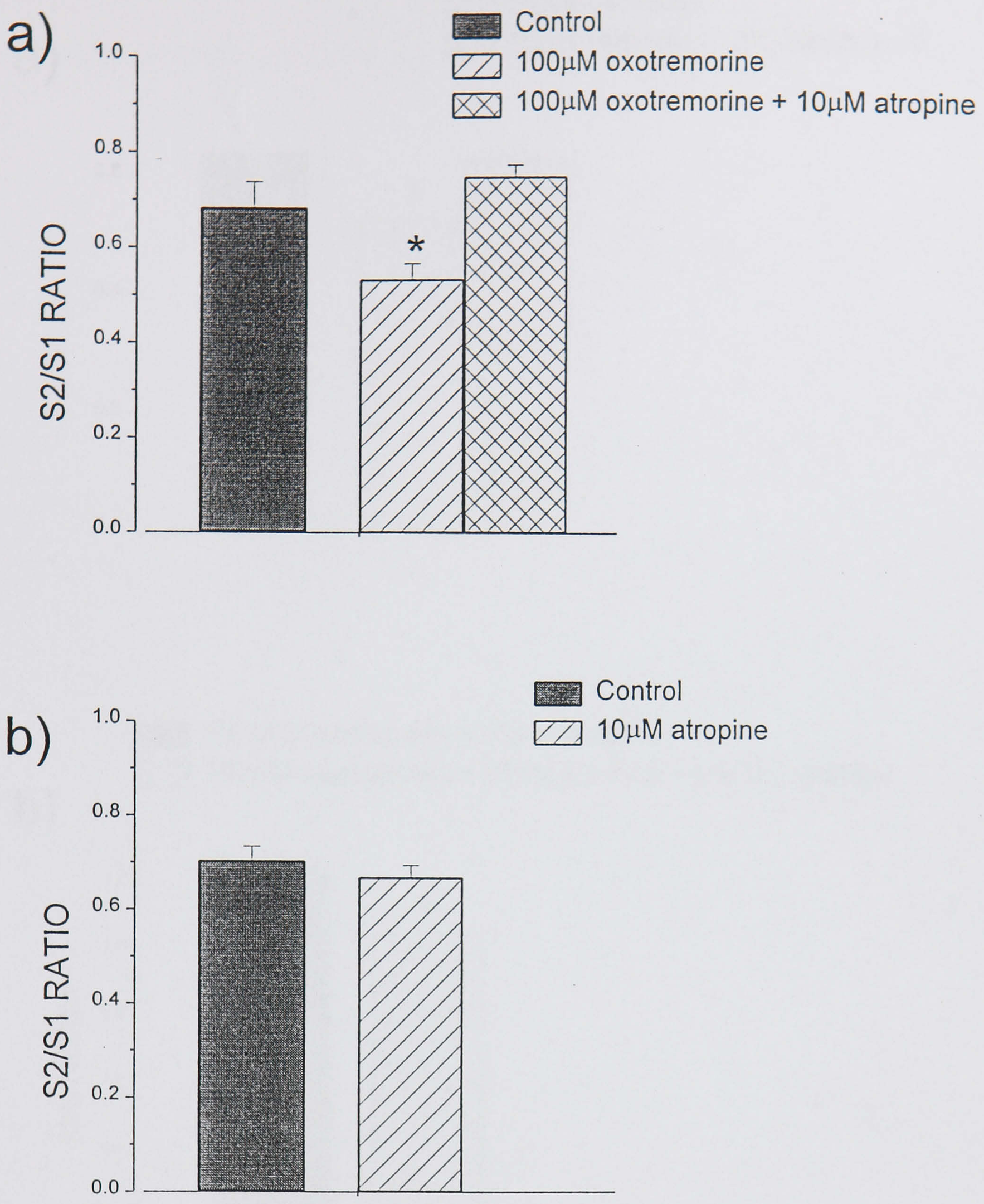


Figure 5.3

The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of atropine on:

a) oxotremorine-induced inhibition of [^{3}H]ACh release

b) K^+-evoked [^{3}H]ACh release

Each column represents the S2/S1 ratio as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) obtained from 3 to 4 experiments; see Results for details. Significant differences compared to the control S2/S1 ratio are denoted by * $P<0.001$.

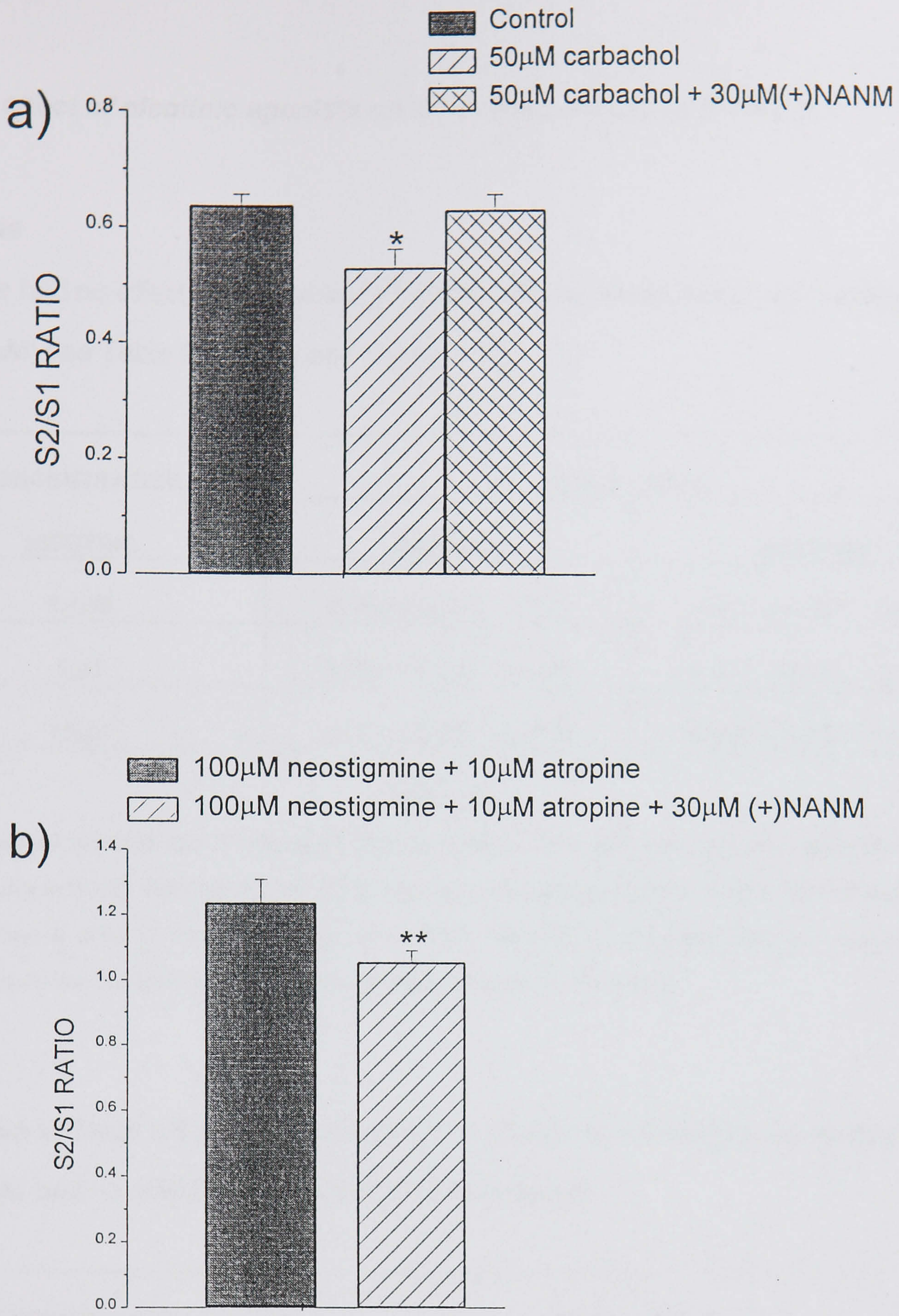


Figure 5.4

The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of (+)NANM on:

a) carbachol-induced inhibition of [^{3}H]ACh release

b) neostigmine and atropine-induced potentiation of [^{3}H]ACh release

Each column represents the S2/S1 ratio as the mean ± SEM of the total number of replicate samples (n) obtained from four experiments; see Results for details. Significant differences compared to the control S2/S1 ratio are denoted by * $P<0.05$.

3. The effect of nicotinic agonists on K^+-evoked release of [^{3}H]ACh

Nicotine

Nicotine had no effect on K^+-evoked [^{3}H]ACh release at concentrations ranging from 0.1-10μM; see Table 5.3 below and Figure 5.5a.

CONCENTRATION NICOTINE	S2/S2 RATIO	
	CONTROL	NICOTINE
0.1μM	0.730 ± 0.018 (N=4)	0.815 ± 0.071 (N=4)
1μM	0.692 ± 0.023 (n=13)	0.742 ± 0.036 (n=14)
10μM	0.687 ± 0.021 (n=13)	0.691 ± 0.037 (n=15)

TABLE 5.3

The effect of nicotine on K^+-evoked [^{3}H]ACh release. The test samples were superfused with nicotine only in the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) observed from four experiments, depending on the analysis of variance; see Methods (Chapter 2) for details.

DMPP

As shown in Table 5.4 below and Figure 5.5b, DMPP at concentrations ranging from 0.1-10μM had no effect on K^+-evoked [^{3}H]ACh release.

CONCENTRATION DMPP	S2/S2 RATIO	
	CONTROL	DMPP
0.1μM	0.725 ± 0.026 (n=13)	0.761 ± 0.037 (n=16)
1μM	0.644 ± 0.023 (n=14)	0.684 ± 0.033 (n=16)
10μM	0.694 ± 0.062 (N=4)	0.762 ± 0.072 (N=4)

TABLE 5.4

The effect of DMPP on K^+-evoked [^{3}H]ACh release. The test samples were superfused with DMPP only in the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) observed from four experiments, depending on the analysis of variance; see Methods (Chapter 2) for details.

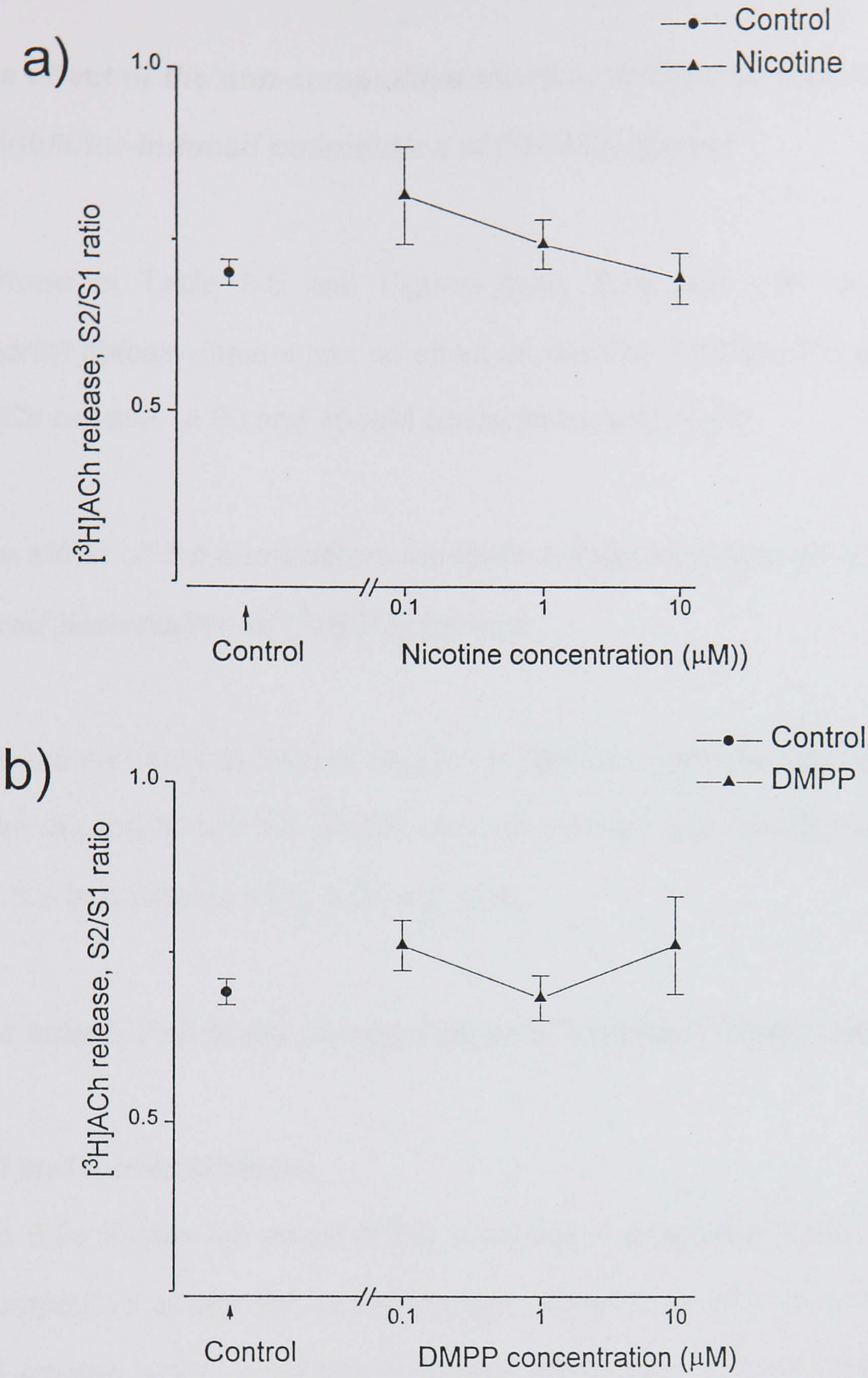


Figure 5.5

The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of nicotinic agonists:

a) nicotine (0.1-10μM)

b) DMPP (0.1-10μM)

The prisms were stimulated twice (S1 and S2) for 2 min and the nicotinic agonists were added only during the S2 stimulation. Each point represents the S2/S1 ratio as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) observed from 4 experiments; see Tables 5.3 and 5.4 for details. The control represents the mean ± SEM of means obtained from all the experiments.

4. The effect of the non-competitive nicotinic antagonist mecamylamine on the ChE-inhibitor-induced potentiation of [^{3}H]ACh release

As shown in Table 5.5 and Figures 5.6a, 5.7a and 5.8a, the non-competive antagonist mecamylamine had no effect on the ChE-inhibitor-induced potentiation of [^{3}H]ACh release, at 30 and 100μM concentrations (P>0.05).

5. The effect of the competitive nicotinic antagonist DHβE on the ChE-inhibitor-induced potentiation of [^{3}H]ACh release

DHβE had no effect at 30 and 100μM concentrations on the potentiation of [^{3}H]ACh release caused by tacrine (30μM) eserine (100μM) and neostigmine (100μM) - see Table 5.6 and Figures 5.6b, 5.7b and 5.8b.

6. The effect of nicotinic antagonists on K^+-evoked [^{3}H]ACh release

DHβE and mecamylamine

Figure 5.9a shows the effect of the competitive antagonist DHβE (100μM) and the noncompetitive antagonist mecamylamine (100μM) on K^+-evoked [^{3}H]ACh release. DHβE caused a 43% increase in release (P<0.001) - control S2/S1 0.727 ± 0.033 n=12; 100μM DHβE S2/S1 1.042 ± 0.042 n=14. The release of [^{3}H]ACh is unaltered in the presence of mecamylamine - control S2/S1 0.727 ± 0.033 n=12; 100μM mecamylamine S2/S1 0.653 ± 0.034 n=15 (P>0.05).

D-tubocurarine and hexamethonium

The effects of two other nicotinic antagonists *d*-tubocurarine and hexamethonium are shown in Figure 5.9b. At 100μM concentrations the non-competitive antagonist hexamethonium had no effect on K^+-evoked [H]ACh release (control S2/S1 0.742 ± 0.037 n=12; 100μM hexamethonium S2/S1 0.794 ± 0.038 n=14; P>0.05) whereas

the competitive antagonist *d*-tubocurarine (100μM) showed a significant increase of 12% ($P<0.05$); control S2/S1 0.742 ± 0.037 n=12; 100μM *d*-tubocurarine S2/S1 0.863 ± 0.036 n=14.

CHE-INHIBITOR (CHE-I)	CHE-I	CHE-I + 30μM MECAMYLAMINE	CHE-I + 100μM MECAMYLAMINE
TACRINE (30μM)	1.158 ± 0.088 (N=4)	0.973 ± 0.082 (N=4)	1.094 ± 0.141 (N=4)
ESERINE (100μM)	1.076 ± 0.043 (n=15)	0.987 ± 0.038 (n=16)	1.143 ± 0.045 (n=16)
NEOSTIGMINE (100μM)	1.221 ± 0.061 (N=4)	1.276 ± 0.074 (N=4)	1.203 ± 0.092 (N=4)

TABLE 5.5.

The effect of the non-competitive nicotinic antagonist mecamylamine on the ChE-inhibitor-induced potentation of K^+-evoked [^{3}H]ACh release.

The test samples were superfused with ChE-inhibitor or ChE-inhibitor and mecamylamine 10 min prior to and during the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) observed from four experiments depending on the analysis of variance; see Methods (Chapter 2) for details.

CHE-INHIBITOR (CHE-I)	CHE-I	CHE-I + 30μM DHβE	CHE-I + 100μM DHβE
TACRINE (30μM)	0.955 ± 0.036 (n=13)	0.864 ± 0.030 (n=14)	0.960 ± 0.042 (n=15)
ESERINE (100μM)	1.099 ± 0.055 (n=15)	1.054 ± 0.040 (n=16)	1.170 ± 0.042 (n=16)
NEOSTIGMINE (100μM)	1.199 ± 0.072 (n=15)	1.121 ± 0.061 (n=15)	1.342 ± 0.066 (n=14)

TABLE 5.6

The effect of the competitive nicotinic antagonist DHβE on the ChE-inhibitor-induced potentation of K^+-evoked [^{3}H]ACh release.

The test samples were superfused with ChE-inhibitor or ChE-inhibitor and DHβE 10 min prior to and during the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of the total number of replicate samples (n) observed from four experiments; see Methods (Chapter 2) for details.

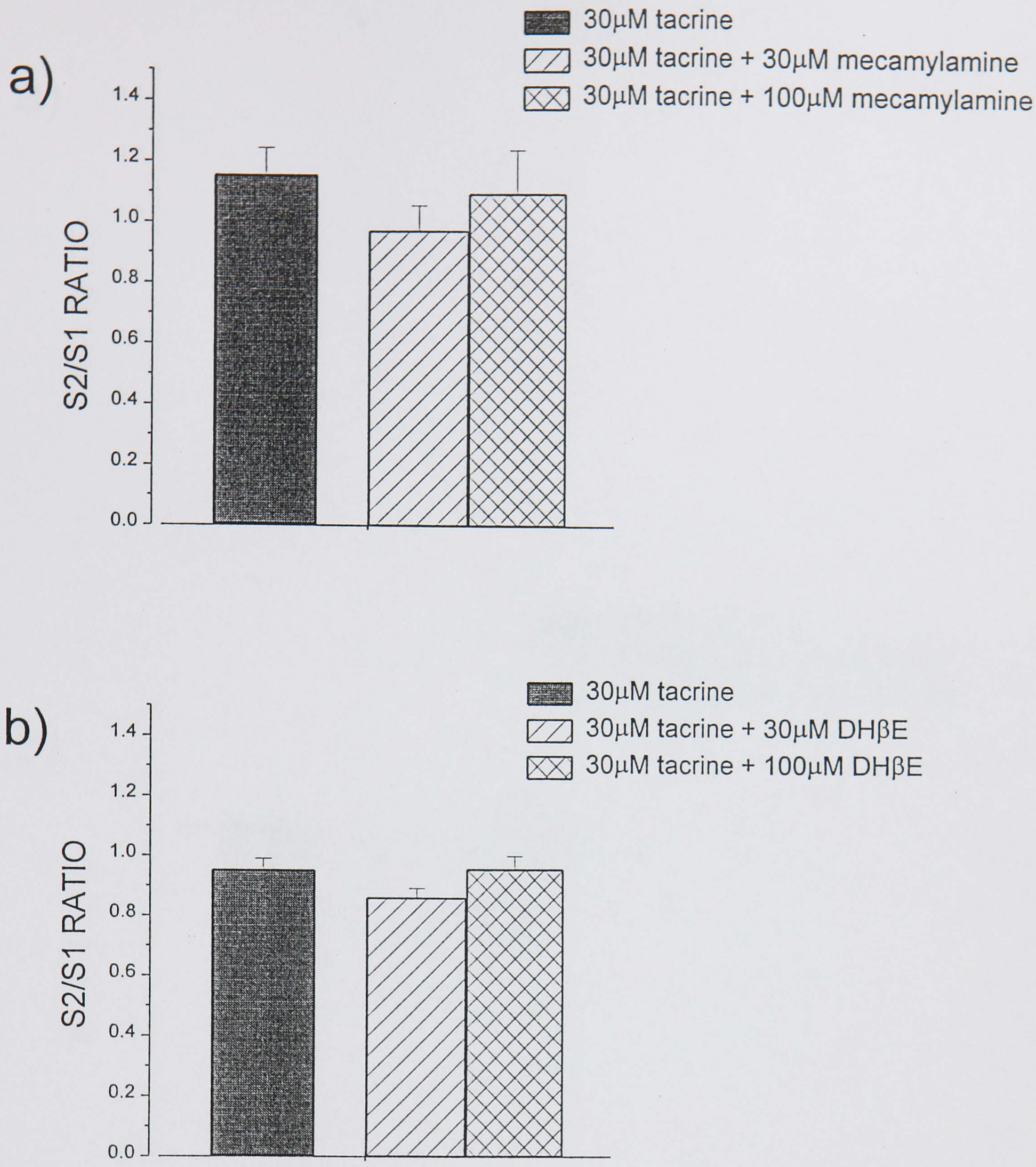


Figure 5.6

The S2/S1 ratios of K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of tacrine with the nicotinic antagonists

a) mecamylamine

b) DHβE

Each column represents the S2/S1 ratio as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) obtained from 4 experiments; see Tables 5.5 and 5.6 for details.

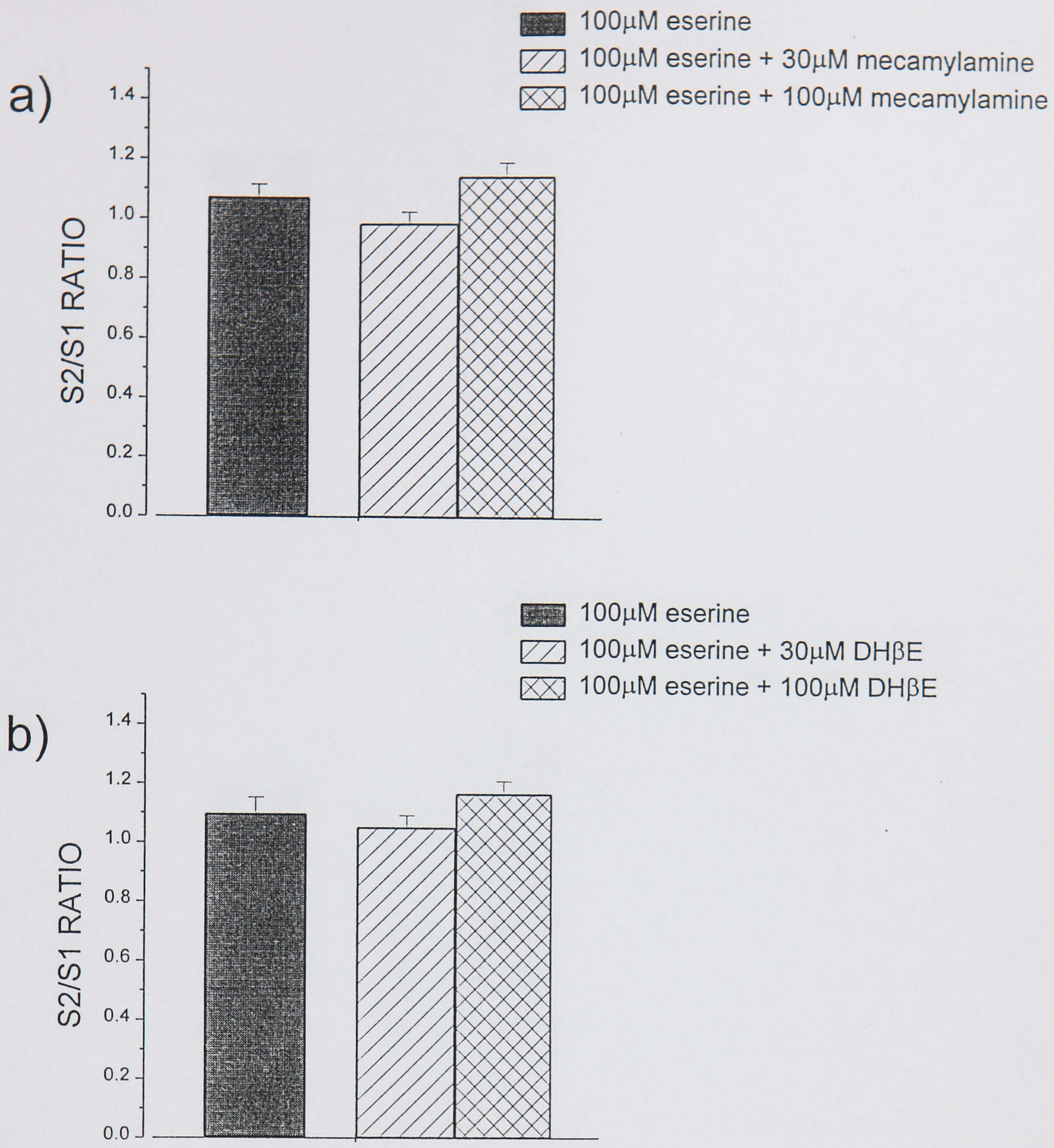


Figure 5.7

The S2/S1 ratios of K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of eserine with the nicotinic antagonists

a) mecamylamine

b) DHβE

Each column represents the S2/S1 ratio as the mean ± SEM of the total number of replicate samples (n) obtained from 4 experiments; see Tables 5.5 and 5.6 for details.

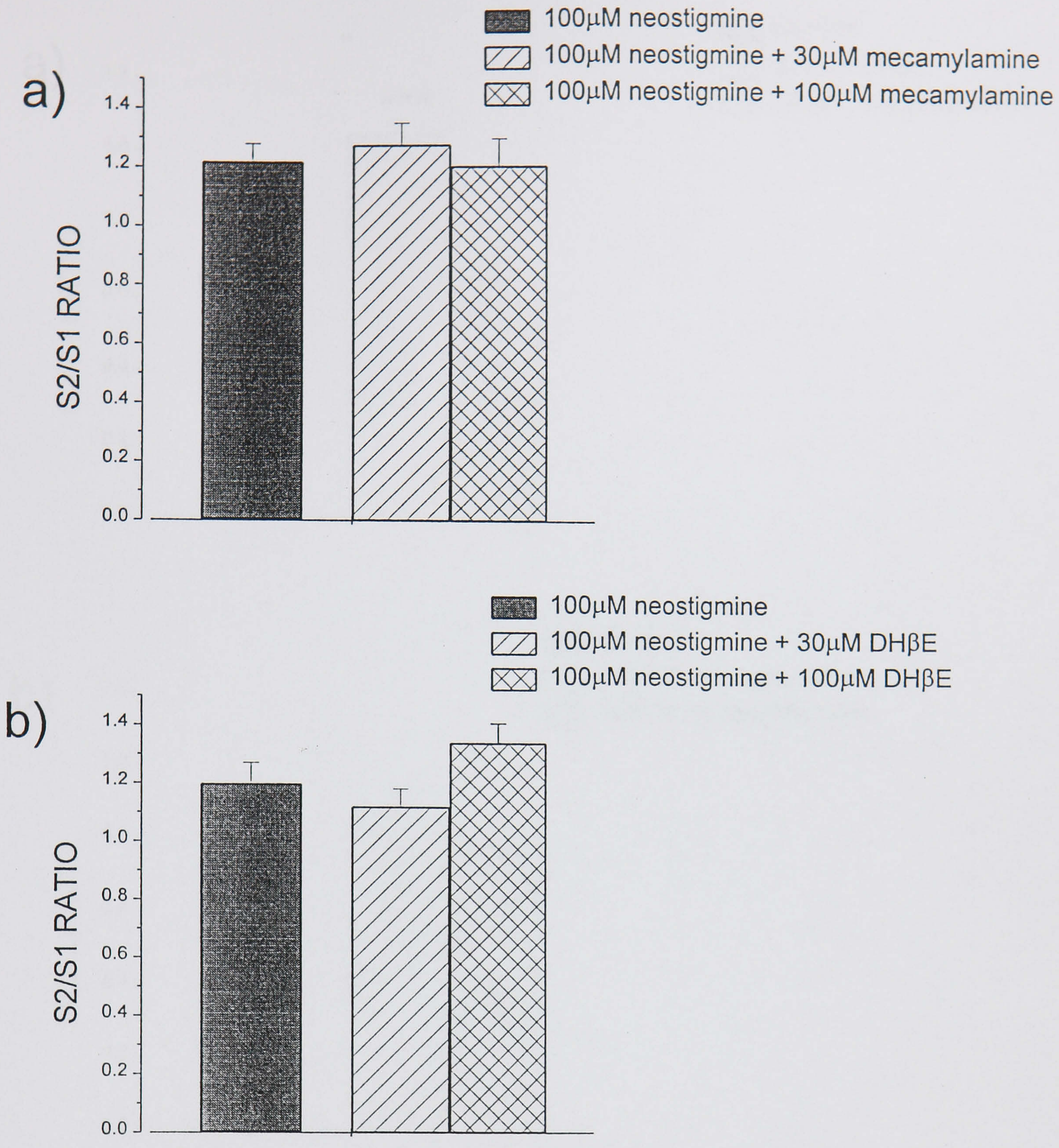


Figure 5.8

The S2/S1 ratios of K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of neostigmine with the nicotinic antagonists

a) mecamylamine

b) DHβE

Each column represents the S2/S1 ratio as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) obtained from 4 experiments; see Tables 5.5 and 5.6 for details.

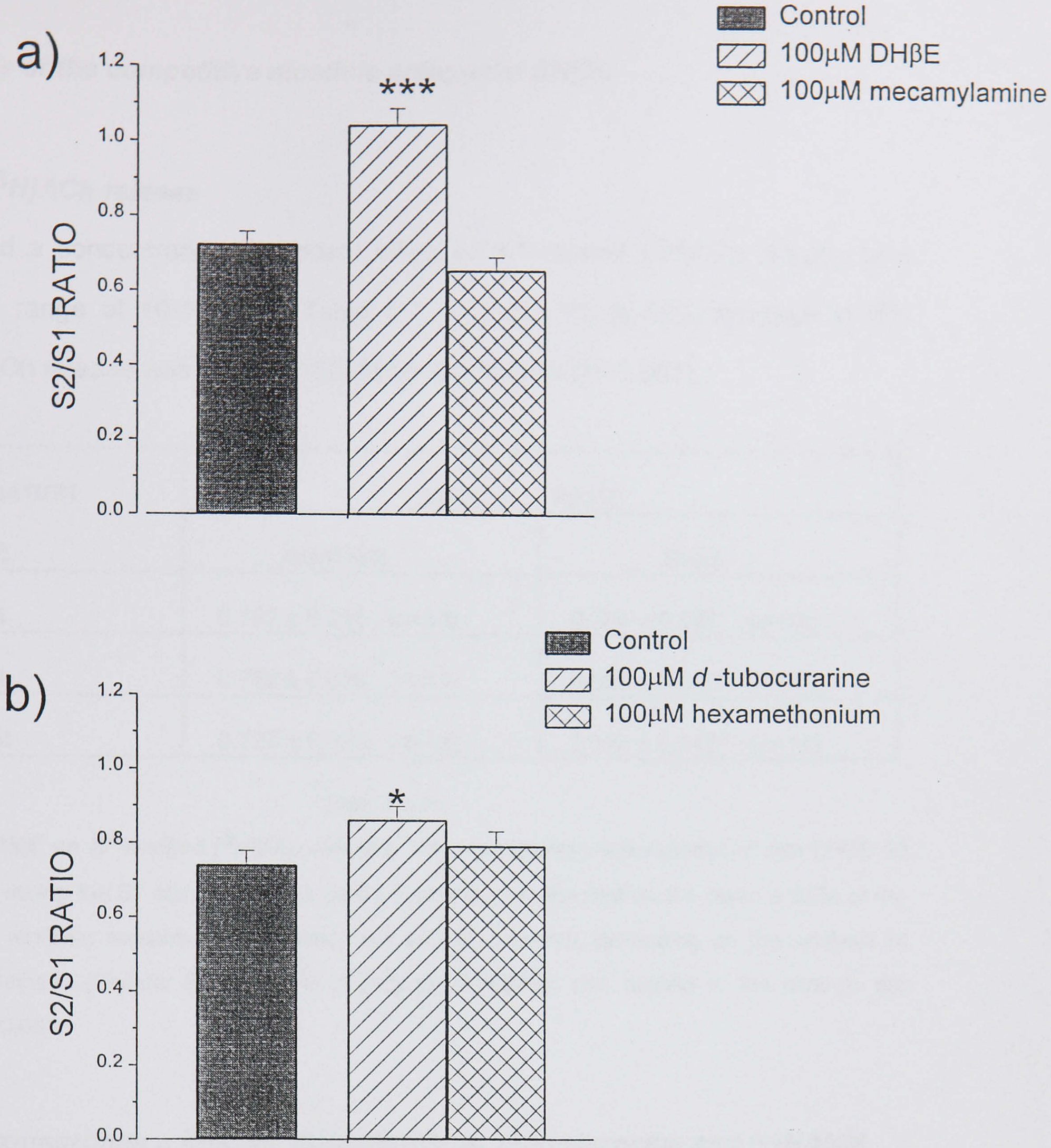


Figure 5.9

The S2/S1 ratios of K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of nicotinic antagonists.

a) DHβE and mecamylamine

b) *d*-tubocurarine and hexamethonium

Each column represents the S2/S1 ratio as the mean ± SEM of the total number of replicate samples (n) obtained from 4 experiments; see Results for details. Significant differences compared to the control S2/S1 ratio are denoted by * P,0.05 and ***P<0.001.

5. The effects of the competitive nicotinic antagonist DHβE:

K^+-evoked [^{3}H]ACh release

DHβE showed a concentration-dependent effect on K^+-evoked [^{3}H]ACh release at a concentration range of 10-100μM - Table 5.7, Figure 5.10. A 43% increase in K^+-evoked [^{3}H]ACh release was seen at 100μM concentrations ($P<0.001$).

CONCENTRATION DHβE	S2/S1 RATIO	
	CONTROL	DHβE
10μM	0.782 ± 0.016 (n=13)	0.786 ± 0.026 (n=14)
30μM	0.782 ± 0.016 (n=13)	0.839 ± 0.050 (n=12)
100μM	0.727 ± 0.033 (n=12)	1.042 ± 0.042* (n=14)

TABLE 5.7

The effect of DHβE on K^+-evoked [^{3}H]ACh release. The test samples were superfused with DHβE 10 min prior to and during the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of the total number of replicate samples (n) observed from four experiments, depending on the analysis of variance; see Methods (Chapter 2) for details. Significant differences with respect to the controls are denoted by * $P<0.001$.

With the benzomorphan σ ligands (±)pentazocine, (+)cyclazocine and (+)NANM

The increase in K^+-evoked [^{3}H]ACh release caused by 100μM concentrations of DHβE was significantly reduced by the benzomorphan σ ligands (±)pentazocine (50μM), and (+)cyclazocine (30μM) but not by (+)NANM (50μM) (Table 5.8, Figure 5.11).

BENZOMORPHAN σ LIGAND	S2/S1 RATIO	
	DHβE	DHβE +σ LIGAND
(±) PENTAZOCINE (50μM)	0.929 ± 0042 (N=6)	0.589 ± 0.050* (N=6)
(+)CYCLAZOCINE (30μM)	0.955 ± 0.059 (n=12)	0.693 ± 037* (n=11)
(+)NANM (50μM)	0.878 ± 0.054 (N=3)	0.911 ± 0.018 (N=3)

TABLE 5.8

The effect of the benzomorphan σ ligands on the DHβE-induced potentiation of K^+-evoked [^{3}H]ACh release. The test samples were superfused with DHβE or DHβE and one of the benzomorphans (±)pentazocine, (+)cyclazocine or ((+)NANM, 10 min prior to and during the S2 stimulation. The S2/S1 values are represented as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicate samples (n) observed from 3 to 6 experiments, depending on the analysis of variance; see Methods (Chapter 2) for details. Significant differences with respect to DHE alone are denoted by * P<0.001.

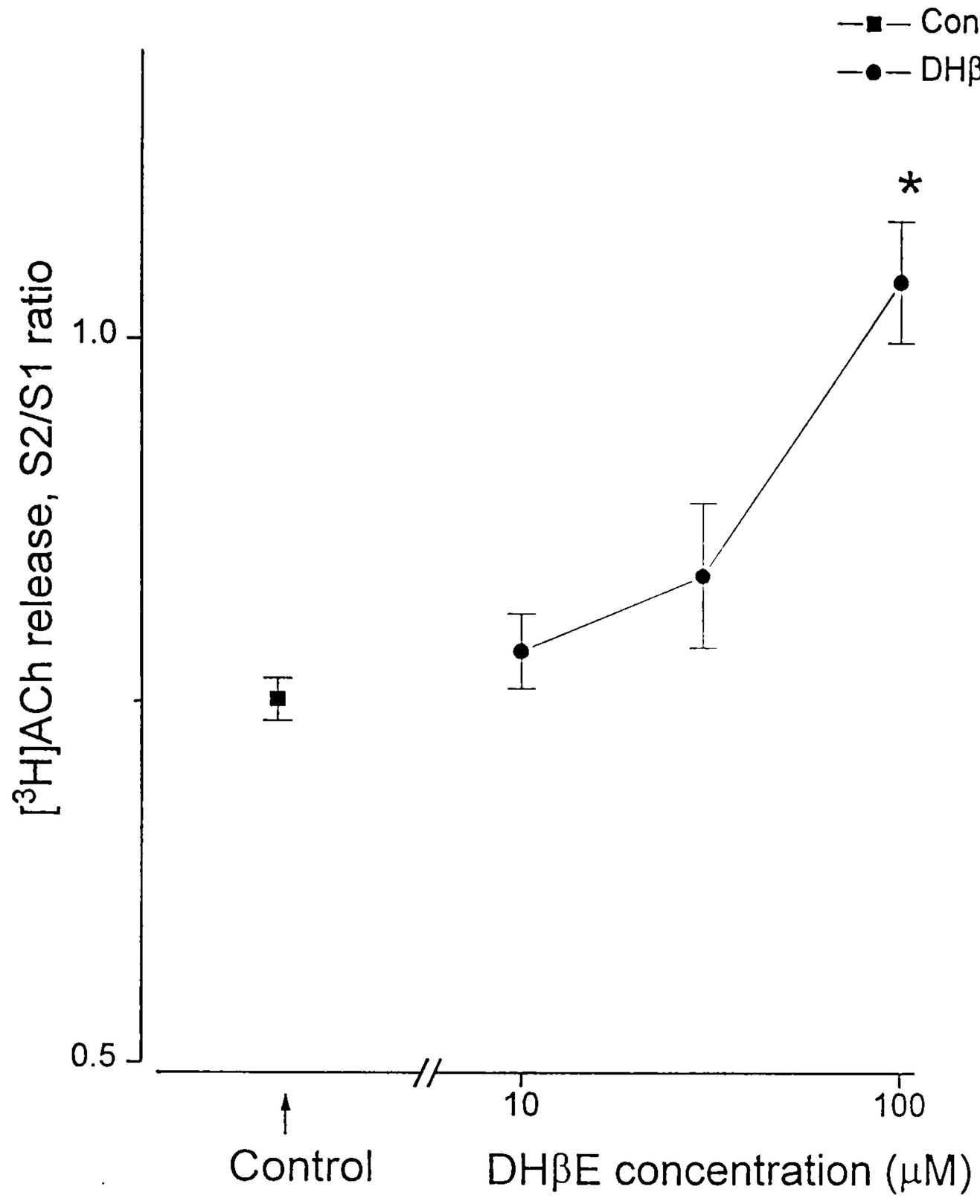


Figure 5.10

The S2/S1 ratios for K^+-evoked release of [^{3}H]ACh from rat hippcampal prisms - the effect of the competitive nicotinic antagonist DHβE (10-100μM).

The prisms were stimulated twice (S1 and S2) for 2 min and DHβE was added to the superfusing medium 10 min prior to and during the S2 stimulation. Each point represents the S2/S1 ratio as the mean ± SEM of the total number of replicate samples (n) observed from 4 experiments; see Table 5.7 for details. The control represents the mean ± SEM of means obtained from all the experiments. Significant differences in the S2/S1 ratio compared to the control group are denoted by *P<0.001.

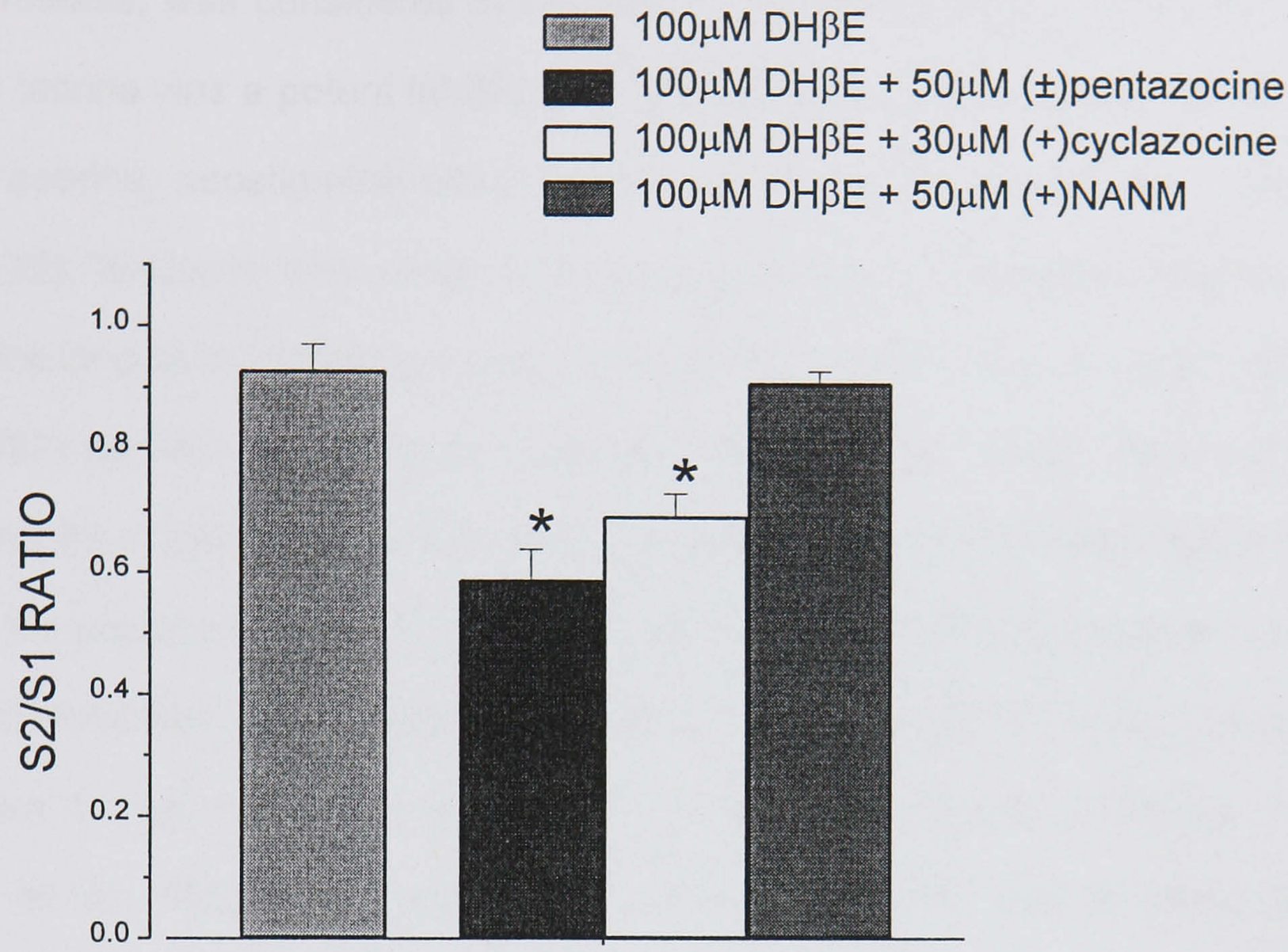


Figure 5.11

The S2/S1 ratios of K^+-evoked release of [^{3}H]ACh from rat hippocampal prisms - the effect of the non-competitive nicotinic antagonist DHβE with the benzomorphan σ ligands (±)pentazocine, (+)cyclazocine or (+)NANM.

Each column represents the S2/S1 ratio as the mean ± SEM of means (N) or the mean ± SEM of the total number of replicates (n) obtained from 3-6 experiments; see Table 5.8 for details. The control represents the mean ± SEM of means obtained from all the experiments (N=6). Significant differences compared to 100μM DHβE S2/S1 ratio are denoted by * $P<0.001$.

DISCUSSION

The possible role of muscarinic receptors in the effects of tacrine and HC-3 on [^{3}H]ACh release, was considered in Chapter 3. Displacement experiments showed that while tacrine was a potent inhibitor of [^{3}H]QNB specific binding, the other ChE-inhibitors eserine, neostigmine and edrophonium and HC-3 were far less effective (Figure 3.23). Evidence also exists that tacrine and the ChE-inhibitors eserine and neostigmine bind at the nicotinic receptor binding site (De Sarno *et al.,* 1989; Nilsson *et al.,* 1987) as well as at the ion channel (Sherby *et al.,* 1985). Moreover the benzomorphan σ ligands (±)pentazocine, (+)cyclazocine and (+)NANM, which were shown in the previous chapter to oppose the potentiation of [^{3}H]ACh release caused by the ChE-inhibitors, also displace [^{3}H]QNB specific binding (Figure 4.11) and are also known to interact with the nicotinic ion channel (King & Aronstam, 1983, Eldefrawi *et al.,* 1982). The aim of this chapter therefore, was to study these muscarinic and nicotinic interactions and their role in the modulation of [^{3}H]ACh release.

Muscarinic interactions

In Figure 5.3b it is shown that atropine itself did not increase K^+-evoked release of [^{3}H]ACh although reports exist that it does so in various brain areas such as the cerebral cortex (Pittel *et al.,* 1990), hippocampus (Nordstrom & Bartfai, 1980; Pohorecki *et al.,* 1988) and striatum (Hadhazy & Szerb, 1977, Weiler *et al.,* 1984). However it has also been reported by Marchi *et al.* (1981) using rat hippocampal synaptosomes and by Szerb and Somogyi (1973) using rat cortical slices, to have no effect on evoked release. One of the reasons for the apparent lack of effect may be due to the different methodologies used - for example the use of slices or synaptosomes, K^+ or electrical stimulation. Furthermore James and Cubeddu (1984) demonstrated that the ability of atropine to increase ACh release was directly

proportional to the intensity of the stimulation. They suggested that although the release of ACh evoked by high rates of stimulation was subject to autoreceptor inhibition, ACh released by low rates, was not. It was reasoned that at higher stimulation rates, the concentration of ACh in the synapse would build up until it exceeded the threshold concentration at which the feedback inhibition mediated by presynaptic receptors would be activated. Antagonists would therefore have more substantial effects at higher stimulation rates, at which the feedback system is more effective. It is possible therefore, that the lack of effect of atropine in the present experiments might be due to inadequate activation of the autoreceptors during the 2 min pulse of 30mM K^+.

Consistent with the above interpretation, atropine in combination with each of the ChE-inhibitors was found to enhance the potentiation of [^{3}H]ACh release caused by these ChE-inhibitors (Figure 5.1). Similar results were first described by MacIntosh and Oborin in 1953, where they reported that atropine, in the presence of a ChE-inhibitor, enhanced the release of ACh from the intact cerebral cortex of the cat (cited in Pohorecki *et al.,* 1988). Since then there have been many supporting observations by other investigators (Molenaar & Polak, 1970; Polak, 1971; Szerb & Somogyi, 1973). It appears that in the presence of ChE-inhibitors, the action of the unhydrolyzed [^{3}H]ACh would result in the stimulation of the muscarinic autoreceptors, thus limiting the potentiation caused by the ChE-inhibitors. Muscarinic antagonists such as atropine would relieve this autoinhibition giving rise to a further enhancement of [^{3}H]ACh release as seen in Figure 5.1.

The existence of presynaptic cholinoceptors that inhibit ACh release has been amply demonstrated using rat brain slices (Szerb & Somogyi, 1973; Hadhazy & Szerb, 1977; Pohorecki *et al.,* 1988) and synaptosomes (Marchi & Raiteri, 1985a; Marchi *et al.,* 1981; Meyer & Otero, 1985; Pittel *et al.,* 1990), either by superfusion (Marchi et al.,1981) or fixed volume incubation (Nordstrom & Bartfai, 1980). In the present

work, it was shown that 10μM atropine effectively reversed the oxotremorine-induced inhibition of [^{3}H]ACh release (Figure 5.3a). Similarly (+)NANM which was demonstrated in Chapter 4 to displace [^{3}H]QNB specific binding (Figure 4.11) is known to be a muscarinic antagonist and was shown to antagonize inhibition of [^{3}H]ACh release by carbachol (Figure 5.4a). These results support the involvement of muscarinic receptors in the autoinhibition of [^{3}H]ACh release and also the reliability of this preparation in the measurement of [^{3}H]ACh release.

Interestingly however, the ChE-inhibitors themselves displace [^{3}H]QNB binding in the order tacrine> eserine = neostigmine = edrophonium (Figure 3.23). Furthermore tacrine has also been reported to be a muscarinic antagonist (see Chapter 3). Thus the possibilty has to be considered that the ChE-inhibitors, as well as potentiating ACh release by inhibiting acetylcholinesterase, might also simultaneously relieve negative feedback inhibition and further enhance potentiation of release ("autoenhancement"). According to the [^{3}H]QNB diplacement data in Figure 3.23 the ChE-inhibitors eserine, neostigmine and edrophonium cause a 40-80% inhibition of [^{3}H]QNB binding at concentrations of 100μM, while tacrine causes a displacement of >90% at 30μM concentrations. Thus it is conceivable that atropine, at saturating concentrations (10μM) is completing the blockade of negative feedback promoted by eserine, neostigmine and edrophonium. However 30μM tacrine alone would be efficient at relieving autoinhibition caused by its own potentiating effect. It is therefore difficult to see how atropine can cause a further enhancement of release comparable to that obtained in the presence of the other ChE-inhibitors. A similar difficulty arises from the observation that atropine relieves the inhibition of [^{3}H]ACh release observed at 100μM tacrine (Figure 5.2a).

It must however be mentioned at this point that the concentration of the ChE-inhibitors tested here, were those that were individually, maximally effective at potentiating [^{3}H]ACh release but not maximally effective at displacing [^{3}H]QNB

(compare Figures 3.3 3.5. 3.7 & 3.9 and Figure 3.23). Moreover eserine, which failed to show a concentration-dependent effect on [^{3}H]ACh release at the concentrations tested, clearly shows a concentration-dependent displacement of [^{3}H]QNB. Thus the functional effects of the ChE-inhibitors are clearly at variance with the binding data observed in Figure 3.23. It might be suggested that this phenomenon is due to the presence of 'spare receptors'. However it has been shown that muscarinic autoreceptors on hippocampal cholinergic nerve terminals do not exhibit such a 'spare receptor' population (Vickroy *et al.,* 1993). Moreoever, the radioligand [^{3}H]QNB is not selective for muscarinic autoreceptors alone, and so its binding and displacement would reflect the entire muscarinic receptor population - pre and post synaptic.

It is possible therefore, that the [^{3}H]QNB binding data is more relevant to muscarinic receptors other than those controlling [^{3}H]ACh release. It has been shown that the affinity of the antagonists atropine, N-methyl-scopolamine and QNB for the presynaptic muscarinic receptors is 10-20 times less than that for postsynaptic receptors (Szerb, 1978). The K_D for QNB reversal of oxotremorine-induced inhibition of [^{3}H]ACh release from hippocampal slices was reported to be approximately 8nM, whereas the K_D for [^{3}H]QNB binding to hippocampal membranes after septohippocampal lesioning was approximately 0.4nM. Thus, in the present experiments which used 0.9nM [^{3}H]QNB, only 10% (approximately) of the receptors controlling ACh release are likely to be radiolabelled in contrast to 70% of the other receptors.

Recent detailed studies on rat hippocampal autoreceptors concluded that the presynaptic sites belong to the M2 (Richards, 1990) and M4 (McKinney *et al.*, 1993) class of muscarinic receptors. Further studies using mRNA analysis and immunoprecipitation by receptor specific antibodies have shown, that these receptors represent a minor component of [^{3}H]NMS binding in the hippocampus - as

little as 10-20% (Vilaro *et al.,* 1993; Ladinsky, 1993). It would therefore appear, that the present [^{3}H]QNB binding data is not a reliable indicator of the presynaptic action of tacrine or the classical ChE-inhibitors eserine, neostigmine and edrophonium. An interpretation therefore of the present data is that the ChE-inhibitors tacrine, eserine, neostigmine and edrophonium cause little or no blockade of presynaptic muscarinic autoreceptors. Atropine, the more potent muscarinic antagonist is therefore able to exert full enhancement of release in the presence of tacrine or the other ChE-inhibitors. That tacrine is ineffective as a muscarinic autoreceptor antagonist is further supported by the observation that at 10μM concentrations it did not reverse the oxotremorine (1μM)-induced inhibition of [^{3}H]ACh release from cortical slices (Potter *et al.,* 1989)

As quoted in Chapter 3, the notion that tacrine is a muscarinic receptor antagonist comes from the observation of its inhibition of carbachol-stimulated PI hydrolysis. However it is known that muscarinic autoreceptors are linked to cAMP (Hulme *et al.,* 1990). Furthermore tacrine is reported to be an appreciable inhibitor of the binding of the M2-selective ligand [^{3}H]AF-DX116 (IC_{50} 1.5μM) to whole membrane preparations from rat forebrain (Nielson *et al.,* 1989). Since the above evidence (Potter *et al.,* 1989) suggests that tacrine is not an autoreceptor antagonist, the implication is that it must be an agonist.

According to the IC_{50} value (1.5μM) reported, tacrine at 30μM concentrations would be expected to completely (~95%) occupy the M2 binding sites and therefore to exert maximum autoinhibition. This is supported by the observation that 10μM atropine, which relieves autoinhibition, enhances [^{3}H]ACh release by 19 and 21% in the presence of 30 and 100μM tacrine. Thus the very pronounced concentration-related decline in [^{3}H]ACh release observed at 30-200μM concentrations of tacrine suggests that a mechanism additional to muscarinic autoinhibition must be

responsible. This lends further support for the role of σ binding sites in the inhibition of [^{3}H]ACh release (Chapter 4) and will be discussed further in Chapter 6.

It is important to note that despite the maximum autoinhibition caused by the agonist action of 30μM tacrine, the overall effect at this concentration is that of potentiation of [^{3}H]ACh release. Thus, as discussed previously (Chapter 3), it is clear that tacrine and the other ChE-inhibitors possess an additional property which is responsible for the marked potentiation of [^{3}H]ACh release. It is known that these ChE-inhibitors also interact with nicotinic receptors and this will be considered presently.

(±)Pentazocine, (+)cyclazocine and (+)NANM also show muscarinic receptor-binding affinities, comparable to the ChE-inhibitors as seen from the [^{3}H]QNB binding data (Figure 4.11) and are known to have muscarinic antagonist actions (Discussion, Chapter 4). However as shown in Chapter 4, (±)pentazocine and (+)cyclazocine actually reduce the potentiation caused by the ChE-inhibitors, rather than enhance it, while (+)NANM has no effect (Figures 4.4 - 4.5, 4.7 - 4.8 & 4.10a). Therefore some other mechanism of action must predominate in their overall effect on [^{3}H]ACh release. As discussed in the next chapter, these σ benzomorphans also exhibit nicotinic receptor binding, which may well be relevant.

Nicotinic interactions

As discussed above, the data presented support the existence of muscarinic autoreceptors mediating negative feedback in the control of ACh release. It therefore seemed reasonable to explore the possibility of nicotinic receptors mediating positive feedback. Evidence favouring the existence of nicotinic autoreceptors has been mentioned in the Introduction to this chapter.

Neither nicotine nor the nicotinic agonist DMPP at concentrations 0.1-10μM affected K^+-evoked release from hippocampal prisms (Figures 5.5a & 5.5b). As nicotine is known to cause desensitization (Beani *et al.*, 1985; Wessler *et al.*, 1986; Wilkie *et al.*, 1993) it is possible that the reason for the observed lack of effect is that the nicotinic receptors are inactivated in this way, even though exposure was restricted to the 2 min period of the S2 stimulation. These results agree with previous reports in which it was shown that nicotine had no effect on K^+-evoked (Lapchak *et al.*, 1989) and electrically stimulated [^{3}H]ACh release (Pohorecki *et al.*, 1989). However Beani *et al.*, (1985) reported that nicotine (18nM-180nM) and the nicotinic agonist cytisine (0.5-50μM), enhanced electrically stimulated release of [^{3}H]ACh from cortical slices prepared from guinea pig brain. Furthermore Wonnacott *et al.* (1989) also showed that nicotine evoked a dose-dependent release of [^{3}H]ACh from hippocampal synaptosomes, which was blocked by the nicotinic antagonists dihydro-β-erythroidine (DHβE) and histrionicotoxin.

Since the reported effects of nicotine on [^{3}H]ACh release have been somewhat diverse, the possibility was considered that positive feedback might be exerted by the build up of unhydrolyzed ACh, caused by the inhibition of acetylcholinesterase. The competitive nicotinic antagonist DHβE and the non-competitive nicotinic channel blocker mecamylamine which have both been shown to block nicotinic responses in the rat hippocampus (Ropert & Krnjevic, 1982; Lukas and Bencherif 1992) were tested against the potentiation of [^{3}H]ACh release caused by the ChE-inhibitors.

Neither DHβE nor mecamylamine had any effect on the potentiation of [^{3}H]ACh release induced by the ChE-inhibitors (Figures 5.6 - 5.8). Interestingly however, DHβE alone, markedly increased control K^+-evoked release of [^{3}H]ACh. A similar effect was observed with *d*-tubocurarine, but to a lesser extent (Figure 5.9a & 5.9b). The nicotinic channel blockers mecamylamine and hexamethonium had no effect alone on K^+-evoked [^{3}H]ACh release (Figure 5.9a & 5.9b).

PAGE 224a, NOTE ADDED AFTER VIVA

Taking into account the statistical uncertainty associated with each of the data points in Figure 5.10, there is only limited support for the possibilty that the DHβE effect saturates with increasing concentration. An interpretation therefore might be that the effect is not mediated by nicotinic receptors. This view is supported by the large concentrations of DHβE required to cause a significant effect (however see page 235a-c). It has been reported that mecamylamine in high concentrations causes the release of DA (Mills & Wonnacott, 1984) possibly by a carrier-mediated mechanism analogous to that by which tyramine causes the release of NA. It may seem feasible therefore that DHβE is having a similar effect in releasing ACh in the present experiments. However if DHβE on the one hand and the ChE-inhibitors on the other act at independent sites it is difficult to see why DHβE did not cause additional release over and above that caused by the ChE-inhibitors (Figures 5.6b-5.8b). To explain this it would have to be additionally speculated that the ChE-inhibitors block the ACh-releasing action of DHβE, possibly by blocking carriers essential to its action.

REFERENCE

Mills, A., & Wonnacott, S. (1984). Antibodies to nicotinic acetylcholine receptors used to probe the structural and functional relationships between brain α-bungarotoxin binding sites and nicotinic receptors.

Neurochem. Int., 6, p249-257.

It is noteworthy that although DHβE alone causes a marked increase, it has no effect over and above that of the ChE-inhibitors (Figures 5.6b - 5.8b). Furthermore, this increase in [^{3}H]ACh release observed with DHβE, was found to be opposed by (+)cyclazocine or (±)pentazocine but not by (+)NANM (Figure 5.11), in a similar manner to that seen with the ChE-inhibitors (Figures 4.4 - 4.5, 4.7 - 4.8 & 4.10). Thus it would appear that the ChE-inhibitors exert their effects via a similar mechanism to that of DHβE. It is suggested that this common mechanism may be nicotinic receptor blockade. Previous reports that tacrine, eserine, neostigmine and DHβE produce a concentration-dependent blockade of nicotine-evoked [^{3}H]DA release from rat striatal synaptosomes (Clarke et al., 1994; Wonnacott *et al.,* 1989; Wonnacott, 1991) strongly support this suggestion.

These results would then suggest that DHβE and the ChE-inhibitors may be acting to relieve negative feedback in some way. DHβE has in fact been reported to block muscarinic receptor-mediated responses (Ropert and Krnjevic, 1982; Bird & Aghajanian, 1976), although in the present experiments this is unlikely to be the case since atropine, the classical muscarinic antagonist, had no effect on K^+-evoked release (Figure 5.3b). Furthermore there are no reports that DHβE has ChE-inhibiting properties and so it is very unlikely that the increase in [^{3}H]ACh release is due to combined effects of muscarinic receptor blockade and autoreceptor activation. It is concluded therefore, that nicotinic receptor blockade is very likely to be responsible for its potentiating effects, implying that nicotinic receptors exert negative feedback on ACh release. This feedback is exerted at control levels of evoked release in the present experiments.

A point worth noting is that while DHβE and *d*-tubocurarine alone, increase K^+-evoked [^{3}H]ACh release, the nicotinic channel blockers mecamylamine and hexamethonium have no effect (Figure 5.9). At first glance these differences might be ascribed to the differences between receptor binding site block and channel

block. However *d*-tubocurarine is also known to be a channel blocker (cited in Lebeda *et al.*, 1982). Moreover it will be recalled that PCP, which is also a non competitive blocker (NCB) rather than a channel blocker (Léna & Changeux, 1993), was shown in Chapter 4 to increase [^{3}H]ACh release (see Figure 4.2).

It might seem reasonable to speculate at this stage that the effects observed with various antagonists, result from their specificities for the nicotinic receptor complex controlling [^{3}H]ACh release. As mentioned in the Introduction to this Chapter, heterogeneity of neuronal nicotinic receptors has been inferred not only from pharmacological studies, but also from electrophysiological and immunohistochemical studies. However it is not immediately clear how nicotinic receptors when activated, might inhibit ACh release - so that blockade by DHβE would, as observed, promote release. Since nicotinic receptors are intrinsic components of cation channels that cause membrane depolarization, they would be expected to bring cholinergic nerve terminals closer to the threshold at which release occurs - i.e to promote release.

However it is well known that in ganglia and at the neuromuscular junction, nicotinic receptor activation leads to failure of subsequent electrophysiological events. For example suxamethonium, a nicotinic agonist, causes failure of muscle action potentials. This is considered to be the result initially, of Na^+ channel inactivation caused by persistent end plate depolarization. There may also be a second phase block caused by nicotinic receptor desensitization.

Similarly it can be speculated that in control K^+-evoked release, elevated extracellular concentrations of ACh might cause sufficient additional depolarization to cause inactivation of some of the voltage-operated Ca^{2+} channels for example, involved in release. Indeed it has been suggested that N-type Ca^{2+} channels, which are known to have relatively fast inactivation kinetics (Scott *et al.*, 1991) may be

inactivated even in the rising phase of K^+-evoked depolarization (Momiyama and Takahashi, 1994). Moreover N-type Ca^{2+} channels are also known to be involved in the release of ACh (Fossier *et al.*, 1994). It might therefore be expected that nicotinic receptor blockade by the ChE-inhibitors or DHβE would prevent the additional depolarization caused by ACh via the nicotinic receptors, thus relieving inactivation of these Ca^{2+} channels. This would then enable these Ca^{2+} channels to be more responsive to K^+-depolarization resulting in an increase in [^{3}H]ACh release.

According to this hypothesis, nicotine or DMPP might be seen as further inactivating Ca^{2+} channels, via additional receptor activation while also introducing a more marked receptor desensitization. This could well be the reason for the observed lack of effect of nicotine or DMPP on [^{3}H]ACh release (Figures 5.5a & 5.5b).

The present results are different to those of Wonnacott *et al.,* (1989), where hippocampal synaptososmes were used (also Wilkie *et al.,* 1993). It was observed that in the absence of depolarizing concentrations of K^+, nicotine evoked the release of [^{3}H]ACh. Furthermore, DHβE blocked these effects (Wilkie *et al.,* 1993; Wonnacott *et al.,* 1989), in marked contrast to the potentiation of release observed with DHβE in the present experiments using 30mM K^+. The difference could well arise from the different depolarizing conditions. As mentioned above, inactivation of some significant fraction of Ca^{2+} channels may accompany K^+ depolarization so that additional depolarization by pharmacological concentrations of nicotine or DMPP may cause further inactivation and a failure of release. In the absence of depolarizing concentrations of K^+, depolarization by nicotinic agonists may be sufficient to evoke [^{3}H]ACh release without extensive Ca^{2+} channel inactivation and receptor desensitization. DHβE would thus block agonist-evoked release of [^{3}H]ACh but promote K^+-evoked release, as observed in the present experiments. Support for this suggestion derives from the observations of Araujo *et al.*, (1988). MCC or nicotine alone evoked the release of [^{3}H]ACh from hippocampal slices but gave no

additional release in the presence of depolarizing concentrations of K^+. DHβE or *d*-tubocurarine again, blocked the agonist-evoked release.

Antagonism of nicotine-evoked release from synaptosomes by DHβE (Wonnacott *et al.,* 1989; Wilkie *et al.,* 1993), indicates that the site of action of the antagonist is the nicotinic autoreceptor located on cholinergic nerve terminals. In these experiments, receptor activation by nicotine is the primary signal by which depolarization and thus release, is brought about. Antagonism of receptor activation by DHβE, must therefore inhibit agonist-evoked release by overcoming the source of depolarization. However where depolarization is primarily independent of receptor activation, as in the presence of 30mM K^+, blockade by DHβE will not inhibit release. Indeed as discussed above, if autoreceptor activation by released ACh contributes to voltage-operated Ca^{2+} channel inactivation, then blockade by DHβE could well result in increase of K^+-evoked release of [^{3}H]ACh.

Physiologically, release *in vivo* is evoked by nerve action potential, which activates voltage-operated Ca^{2+} channels in the active zones of the nerve terminal (Smith & Augustine, 1988). However Ca^{2+} channel activation by ACh release would presumably be too brief to lead to significant inactivation of Ca^{2+} channels. It seems unlikely therefore, that DHβE would normally potentiate release *in vivo* in the manner demonstrated in the present experiments. However it is possible that the increased synaptic concentrations of ACh, caused by the ChE-inhibitors would contribute to and prolong nerve terminal depolarization, and thus lead to some Ca^{2+} channel inactivation. Under these circumstances, autoreceptor blockade by the ChE-inhibitors themselves, could well contribute to the potentiation of ACh release *in vivo*.

Interestingly Nordberg *et al.,* (1985) reported that eserine or tacrine potentiated K^+-evoked release of [^{3}H]ACh from post mortem Alzheimer brain tissue. Relevant here, DHβE, mecamylamine or *d*-tubocurarine blocked this potentiation in marked contrast

to the effects reported in the present chapter. A similar result was obtained with mecamylamine in hippocampal slices from rats treated with the cholinotoxin AF64A (ECMA) (Potter & Nitta, 1993). However it must be borne in mind that evoked release of [^{3}H]ACh from post mortem and biopsy Alzheimer tissue is significantly reduced, compared to control tissue (Nilsson *et al.,* 1987; Nordberg *et al.*, 1989; Giacobini, 1990; see Chapters 1 & 3) and may not therefore contribute significantly to Ca^{2+} channel inactivation.

Be this as it may, the data presented in this chapter can be explained by the existence of both nicotinic and muscarinic receptors on the cholinergic nerve terminals. It appears that activation of both these types of receptors by release of ACh leads to inhibition of release. Nicotinic receptors are activated by control K^+-evoked release and muscarinic receptors are activated only during potentiation of release. Nicotinic receptors appear to underlie potentiation by the ChE-inhibitors and DHβE. Muscarinic receptors limit this potentiation and underlie the enhancement of potentation caused by atropine. This hypothesis is considered further in the final chapter.

CHAPTER 6

Conclusions

It was suggested in the previous chapter that the ChE-inhibitors and DHβE probably exert their effect on K^+-evoked ACh release by interacting with nicotinic receptors located on the nerve terminal. It appears as though these nicotinic receptors exert a negative feedback effect which is blocked by the drugs mentioned. The hypothesis was developed that the mechanism underlying the negative feedback effect is that of inactivation of Ca^{2+} channels involved in release. Thus blockade of the nicotinic receptors by DHβE or ChE-inhibitors allows Ca^{2+} channels to reactivate and to respond to K^+-depolarization, so that [^{3}H]ACh release is potentiated.

Nevertheless the effects of (+)cyclazocine and (±)pentazocine remain unexplained. These compounds which are better known as σ benzomorphans, oppose the potentiation caused by the ChE-inhibitors, but have no effect by themselves on K^+-evoked (control) release (see Chapter 4). However cyclazocine, pentazocine and NANM are also known to interact with nicotinic receptors and have been shown to displace [^{3}H]perhydrohistrionicotoxin (IC_{50}~5μM for all three benzomorphans) (King & Aronstam, 1983) and [^{3}H]PCP from nicotinic channel sites (K_i values of 1.2 and 0.9μM for cyclazocine and NANM respectively) (Eldefrawi *et al.*, 1982). It is quite possible therefore, that nicotinic receptor blockade contributes to the observed effects of (+)cyclazocine and (±)pentazocine on [^{3}H]ACh release. However since (+)cyclazocine and (±)pentazocine inhibit only potentiated release, while DHβE potentiates only K^+-evoked release, some other mechanism of action would appear to intervene.

It will be recalled that in Chapter 4, inhibition of control K^+-evoked [^{3}H]ACh release by haloperidol, DTG and tacrine was ascribed to an action at σ binding sites. It is plausible therefore that the observed effects of (+)cyclazocine and (±)pentazocine are the net result of σ–mediated inhibitory action and the nicotinic autoreceptor action which causes potentiation of [^{3}H]ACh release. Since these two effects oppose each other,(+)cyclazocine and (±)pentazocine would have little or no effect on control

K^+-evoked [^{3}H]ACh release, while DHβE lacking σ activity would cause potentiation, as observed (Figures 4.3, 4.6, 5.9a & 5.10). Furthermore, if the nicotinic autoreceptors are already occupied by the ChE-inhibitors such as tacrine, eserine, neostigmine, edrophonium or by DHβE, the inhibitory σ activity of (+)cyclazocine or (±)pentazocine would then be unmasked as observed in Figures 4.4 - 4.5 & 4.7 - 4.8.

Since haloperidol and DTG markedly inhibited control K^+-evoked [^{3}H]ACh release (Figures 4.14 & 4.17) it is not unreasonable to expect (+)cyclazocine or (±)pentazocine to show significant inhibitory effects under the same conditions, despite an opposing effect at the nicotinic autoreceptors. There is evidence of the σ inhibitory action predominating, in the effect of (+)cyclazocine on control K^+-evoked release, which was significantly inhibited at higher concentrations of (+)cyclazocine (Figure 4.6). Similarly, the effect of (±)pentazocine on the potentiation caused by eserine (Figure 4.4b) and DHβE (Figure 5.11), reduced the S2/S1 ratio to levels below control.

Interestingly tacrine at low concentrations potentiates K^+-evoked release but causes inhibition at greater concentrations (Figure 3.3). It would appear therefore that the anticholinesterase and nicotinic autoreceptor blocking properties initially predominate and that the σ inhibitory activity becomes evident only at greater concentrations. As was demonstrated, the [^{3}H]pentazocine displacement curves showed that although weak, tacrine was effective at displacing this σ ligand whereas the classical ChE-inhibitors showed negligible σ binding (Figure 4.18). The lack of an inhibitory phase at higher concentrations of the classical ChE-inhibitors (in contrast to tacrine) supports this suggestion.

The lack of effect of (+)NANM however, on potentiated [^{3}H]ACh release, remains unexplained. It was observed that this "prototypic σ ligand" did not inhibit the neostigmine-potentiated [^{3}H]ACh release in a similar manner to that exhibited by

(+)cyclazocine and (±)pentazocine (Figure 4.10a). Although (+)NANM and (+)cyclazocine have similar affinity for σ binding sites (Walker *et al* 1990), 30μM (+)NANM had no significant effect on potentiated [^{3}H]ACh release, while 30μM (+)cyclazocine caused marked inhibition (Figures 4.10a & 4.8).

However, from a number of reports it appears that (+)NANM also has muscarinic receptor blocking properties (see Introduction and Discussion in Chapter 4). Indeed it was shown in Chapter 5, Figure 5.4a, that (+)NANM reverses the carbachol-induced inhibition of [^{3}H]ACh release indicating an appreciable muscarinic autoreceptor blockade. Thus it may be assumed that (+)NANM would have an atropine-like enhancing effect on potentiation over and above that caused by the nicotinic action of neostigmine (or the other ChE-inhibitors). Furthermore this additional atropine-like enhancement would oppose the inhibitory σ effect of (+)NANM so that overall, release would remain similar to the ChE-inhibitor-potentiated levels. This is in fact what was observed (Figure 4.10a) and further supported by the finding that in the presence of atropine, neostigmine-potentiated [^{3}H]ACh release was clearly inhibited by (+)NANM (Figure 5.4b). It seems that the independent block of the muscarinic autoreceptors by atropine unmasks the inhibitory σ effect of (+)NANM. In comparison, any muscarinic autoreceptor blocking properties of (+)cyclazocine or (±)pentazocine would appear to be weaker so that the σ inhibitory effect is unopposed. Thus as observed, inhibition by (+)cyclazocine or (±)pentazocine of the potentiation caused by neostigmine, the classical ChE-inhibitors or DHβE is clearly significant compared to the effect of (+)NANM.

Although an additional muscarinic autoreceptor action might explain the lack of effect of (+)NANM on potentiated release, it cannot explain the lack of effect on control K^+-evoked release since atropine itself had no effect on controls (Figure 5.3a). (+)NANM would therefore appear to also act on nicotinic autoreceptors. Indeed as mentioned in Chapter 5 (Introduction), NANM has been shown to have non competitive actions

on nicotinic receptors (King & Aronstam 1983). Like (+)cyclazocine or (±)pentazocine therefore, the nicotinic autoreceptor-mediated potentiation opposes σ–mediated inhibition, leaving control K^+-evoked release unchanged.

In Chapter 4 it was shown that the σ ligand (+)3PPP behaved very much like (+)NANM in that it had no effect on control K^+-evoked release or on neostigmine-potentiated [^{3}H]ACh release (Figures 4.16a & b). According to the above argument these results could be explained if (+)3PPP were also a muscarinic and nicotinic autoreceptor blocker. Interestingly it has been reported that (+)3PPP inhibits carbachol-induced contractions of the guinea pig ileum (Vargas & Pechnick, 1991) although there is no evidence in the literature to suggest muscarinic or nicotinic autoreceptor action. On the basis that muscarinic and nicotinic autoreceptor effects oppose σ binding site-mediated inhibition, the marked inhibition of [^{3}H]ACh release by DTG or haloperidol would imply that neither of these drugs have significant muscarinic nor nicotinic autoreceptor-mediated effects. Indeed it is well reported that haloperidol and DTG are very weak antagonists of this type (Hudkins & DeHaven-Hudkins, 1991; Vargas & Pechnick, 1991).

Relevant to this discussion is the nature of the σ effects possessed by the various drugs discussed above. In Chapter 4 it was concluded that the potentiation of [^{3}H]ACh release could not be related to a σ1 effect since the ChE-inhibitors had negligible [^{3}H]pentazocine displacing effects. On the basis of the direct inhibitory effects of haloperidol and DTG, it was tentatively suggested that σ2 activity might be responsible for this effect. However the recognition that (+)cyclazocine and (±)pentazocine and (+)NANM (and possibly (+)3PPP) also have inhibitory effects (albeit masked by presynaptic cholinoceptor activity) would suggest that the inhibition is mediated by σ1 binding activity. These agents are known to be selective for σ1 binding sites whereas DTG and haloperidol are known to be less selective with appreciable σ1 as well as σ2 activity. However as commented in Chapter 4, there is a

marked discrepancy between the micromolar concentrations of σ ligands causing inhibition of [^{3}H[ACh release observed in this study and the reported nanomolar IC_{50} and K_D values for displacement of σ1 or σ2 ligands.

Nevertheless a third low affinity σ binding in the micromolar concentration range has recently been described (Wu *et al.*, 1991). It is possible therefore that this binding underlies the inhibitory effects seen in the present studies of [^{3}H]ACh release. This low affinity σ binding site was identified with the modulation of tonic K^+ currents (in NCB20 cells). Significantly tacrine, demonstrated in the present work to be a σ ligand, and haloperidol, which both inhibit [^{3}H]ACh release strongly (Figures 3.3 & 4.14) also modulate K^+ channels (see Chapter 3) (Morio *et al.*, 1994; Marsh *et al.*, 1990; Halliwell & Grove 1989). It is suggested therefore that σ binding sites may be part of a signal transduction pathway linked to K^+ channels.

A final aspect of this study that must be mentioned is that the experimental work concentrated entirely on the young, adult (200-300g) rat brain, whereas drug therapy would have to be more geared towards the partially degenerated, aging brain in order to be clinically successful. As Nordberg *et al.*, (1989) demonstrated, the effect of eserine and tacrine were quite different when compared in control and Alzheimer brain tissue (see Chapter 3, introduction). A further line of investigation and perhaps more relevant to the treatment of Alzheimers might be [^{3}H]ACh release from the AF64A lesioned rat. Indeed preliminary work of this kind has recently been reported (Potter & Nitta, 1993).

It may be possible to evaluate diffusion barriers of this kind by studying the equilibration of inert markers like mannitol or inulin into, and their elution from, prisms of different sizes. Diffusion barriers may well explain why the classical ChE-inhibitors, DHβE and the σ ligands had to be used throughout this work at concentrations greater than pharmacologically accepted values. In vivo microdialysis studies would shed more light on the relevance of effective concentrations of tacrine and the other drugs. Indeed studies of this type have shown that tacrine does not release monoamines at therapeutic doses (Baldwin et al., 1991 referenced in this thesis).

b) Relevance of the drug actions studied in this thesis to clinical situations:

According to Nielson's binding data (Nielson et al., 1989, referenced in this thesis) clinical concentrations of tacrine (0.2-0.6μM) would cause only 13-32% occupation of M2 autoreceptors. Thus at clinically relevant concentrations, tacrine is unlikely to exert strong muscarinic autoreceptor effects of the kind discussed here (Chapter 5).

In view of the possible diffusion barriers discussed above, the nicotinic receptor-mediated potentiation of ACh release by the ChE-inhibitors and by DHβE, observed in the present work (Chapter 5) might well occur at much lower concentrations than suggested by the effective superfusion concentrations. As discussed (page 228) increased synaptic concentrations of ACh, caused by inhibition of ChE, might well contribute to and prolong nerve terminal depolarization, thus leading to some Ca^{2+} channel inactivation. Under these circumstances autoreceptor blockade by clinical concentrations of the ChE-inhibitors might contribute to the potentiation of ACh release in vivo.

A similar arguement based on diffusion barriers can be made about the relevance of the σ effects discussed in Chapter 4. Sigma-mediated inhibition of ACh release by tacrine at superfusion concentrations greater than 30μM might well translate into concentrations in vivo close to the clinical range.

PAGE 235a,b,c **NOTE ADDED AFTER VIVA**

Relevance of the present work to the treatment of Alzheimer's disease

a) Concentrations effective in the tissue superfusates:

In the present study it was only possible to demonstrate that tacrine had effects on [^{3}H]ACh release at concentrations of 10μM and above. Although these concentrations are in accord with those reported by other workers using similar techniques they contrast with concentrations achievable clinically. These are in the range 0.2-0.6μM in plasma. The IC_{50} for ChE inhibition by tacrine however, is approximately 0.24μM (Atack et al., 1989, this thesis page 126). It is possible therefore that if clinical concentrations could be closer to those in the present study a greater inhibition of the enzyme and possibly a more pronounced therapeutic effect would be achieved. There would appear to be a clinical need for an alternative to tacrine that can be used at concentrations greater than the IC_{50} without causing serious side effects.

However it may be that prism-superfusion techniques require exaggerated drug concentrations to cause an observable effect on [^{3}H]ACh release. Thus there may be marked diffusion barriers between superfusing drug solutions and the intercellular space within the tissue, even though the prism size used is well within the range in which the penetration of oxygen is adequate (this thesis page 78).

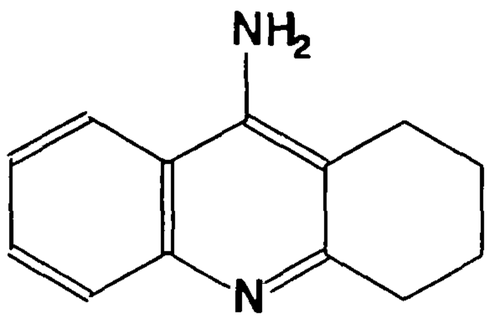
Incubation for 20 min with concentrations of oxotremorine ranging from 0.01-0.1μM inhibited ACh release in a dose-dependent manner from 0.5mm hippocampal prisms stimulated with 35mM K^+ for 5 min (Benishin, 1990, referenced in this thesis). In this present study 12 min incubation with 100μM oxotremorine was required to cause similar inhibition (Fig. 5.3b) using 2 min stimulations with 30mM K^+. It seems therefore that there may be marked concentration / time dependent diffusions involved in K^+-evoked release from tissue prisms and its susceptibility to superfusing drugs.

In considering the relevance of the present results to Alzheimer therapy it should be remembered that the effects were obtained using fresh brain samples from young adult rats. In samples from Alzheimer patients levels of ACh release are known to be reduced so that presynaptic feedback could well be be less prominent. Thus although the inhibition of ChE and the σ effects discussed here remain relevant the nicotinic effects may not be so. The use of lesioned animals therefore would further this line of research.

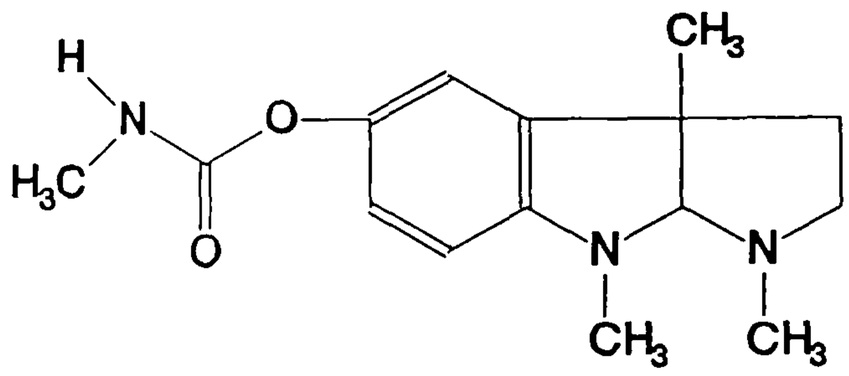
The effect of tacrine and the other ChE-inhibitors, as well as DHβE, on [^{3}H]ACh release from rats partially lesioned with ECMA, and measured using in vivo microdialysis and in vitro methods would be a useful direction to take. Indeed a preliminary experiment of this kind was recently reported (Potter & Nitta, 1993, referenced in this thesis). The use of transgenic mice (Chapter 1) will also be crucial in determining the effect of plaques on ACh release in Alzheimer's disease and suggests a more relevant model with which to test the drug effects described in this thesis.

Taken together it is clear that the measurement of brain ACh release is important in determining the overall efficacy of cholinomimetics such as tacrine and the other ChE-inhibitors. However it is now clear that the relevance of the concentrations as well as the physiological and pathological conditions must also be recognized when developing the "ideal" screening system for cholinomimetic efficacy. Cholinomimetics may also be in the form of muscarinic autoreceptor antagonists which enhance ACh release in the presence of ChE-inhibitors as demonstrated in this study (Chapter 5). As mentioned previously in the Introduction (Chapter 1) nicotinic compounds have not been extensively tested although recent research into allosteric modulation of the nicotinic receptor has begun to renew interest in this area.

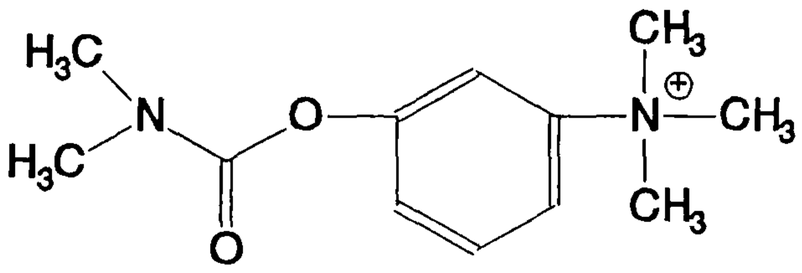
APPENDIX



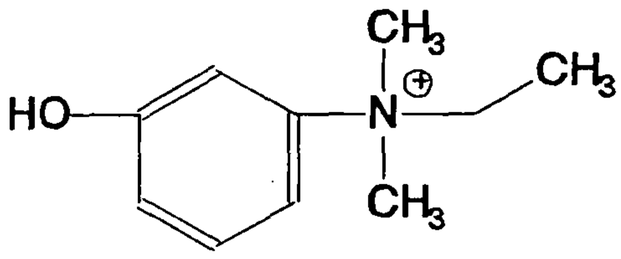
Tacrine



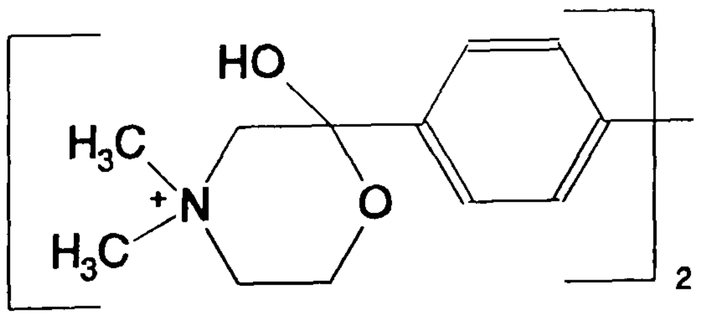
Eserine



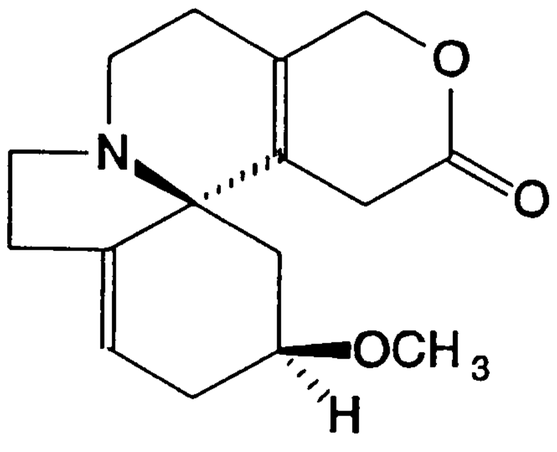
Neostigmine



Edrophonium

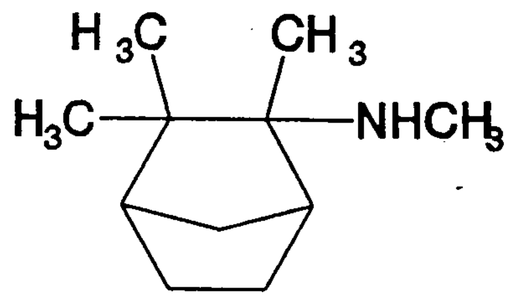


Hemicholinium – 3



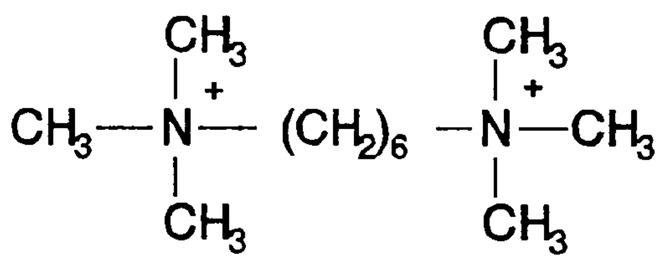
Dihydro- β-erythroidine

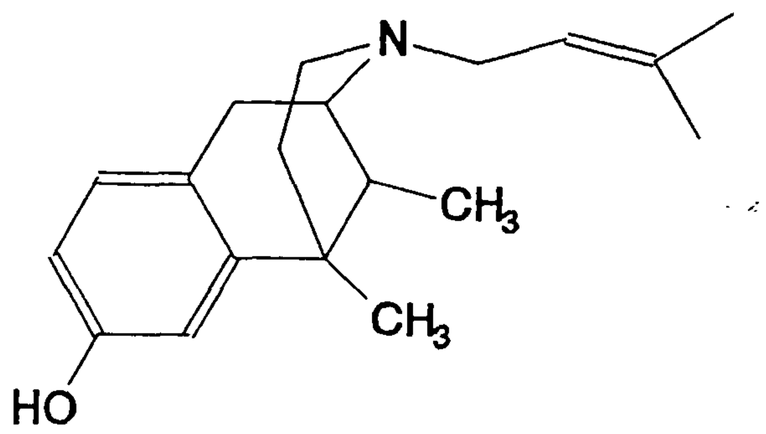
Mecamylamine



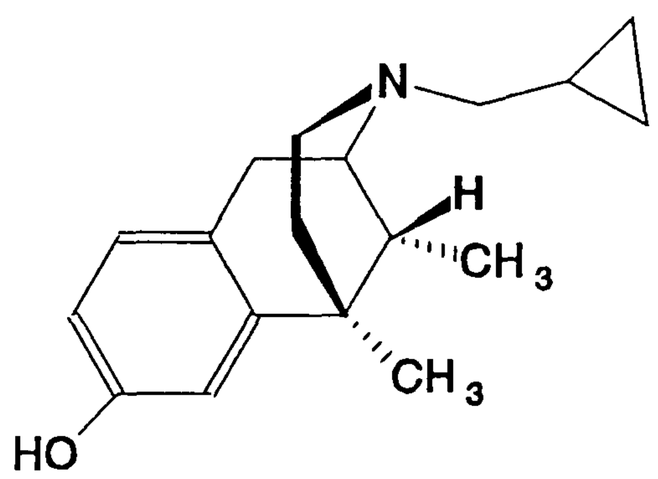
α -Tubocurarine

Hexamethonium

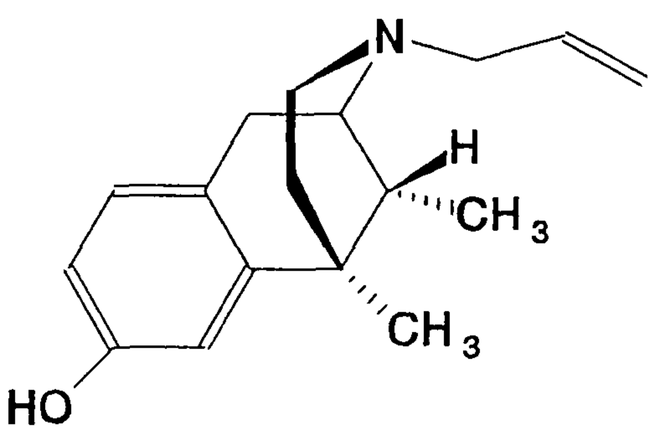




(±) Pentazocine

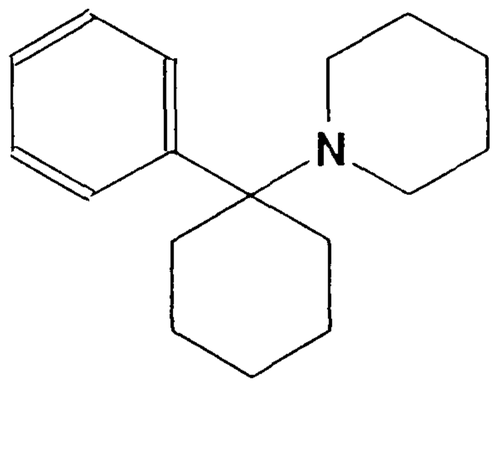


(+) Cyclazocine



(+) N-Allylnormetazocine

(+) NANM



Phencyclidine
(PCP)

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